# ISOLATION AND IDENTIFICATION OF NONTUBERCULOUS MYCOBACTERIA IN SOIL AND WATER SAMPLES FROM AROUND KAMPAR, MALAYSIA

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# ISOLATION AND IDENTIFICATION OF NONTUBERCULOUS MYCOBACTERIA IN SOIL AND WATER SAMPLES FROM AROUND KAMPAR, MALAYSIA

By

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## ABSTRACT

# ISOLATION AND IDENTIFICATION OF NONTUBERCULOUS MYCOBACTERIA IN SOIL AND WATER SAMPLES FROM AROUND KAMPAR, MALAYSIA

**TING CHEE SIONG**

Nontuberculous mycobacteria (NTM) are mycobacteria that do not cause tuberculosis and leprosy. Unlike the pathogenic *Mycobacterium tuberculosis* and *M. leprae*, NTM become opportunistic pathogens only under some predisposing conditions. Although NTM are ubiquitously distributed in the environment, isolation by culture is difficult due to their slow growth rate and the presence of large populations of rapid-growing microbiota. This project was carried out to isolate and identify NTM in soil and water samples collected from several locations around Kampar. Samples were initially decontaminated with a combination of 3% w/v sodium dodecyl sulfate, 4% w/v sodium hydroxide, and 2% w/v cetrimide, in which contamination was successfully controlled for some of them. Decontaminated samples were then cultured on Löwenstein-Jensen media. The Ziehl-Neelsen acid-fast staining was performed on bacterial colonies with morphology and growth rates that were suggestive of those for mycobacterial species. After that, the isolated mycobacteria were identified to the species level based on their phenotypic characteristics and 16S ribosomal DNA sequences. Overall, only six out of 18 samples were positive

for acid-fast bacilli (AFB), and these were from three sampling locations. The remaining samples yielded no microbial growth or their cultures were heavily contaminated. All three soil samples from a cow farm were positive for AFB and the isolates were successfully identified to be that of *M. fortuitum*, *M. senegalense*, and *M. porcinum*, respectively. Two water samples from an ex-tin-mining lake were also AFB-positive but the isolates were not speciated due to overgrowth of non-acid-fast organisms. Interestingly, *Nocardia* sp., an actinobacterial species closely related to mycobacteria, was isolated from one water sample from another ex-tin-mining lake. The isolated NTM species have been reported to be clinically relevant and their health impacts on potentially susceptible human and animal hosts in the location of study were briefly assessed.

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## DECLARATION

I hereby declare that the project is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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(Ting Chee Siong)

**APPROVAL SHEET**

This project report entitled "**ISOLATION AND IDENTIFICATION OF NONTUBERCULOUS MYCOBACTERIA IN SOIL AND WATER SAMPLES FROM AROUND KAMPAR, MALAYSIA"** was prepared by TING CHEE SIONG and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Biotechnology at Universiti Tunku Abdul Rahman.

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**PERMISSION SHEET**

It is hereby certified that **TING CHEE SIONG** (ID No: **10ADB01399**) has completed this final year project entitled **"ISOLATION AND IDENTIFICATION OF NONTUBERCULOUS MYCOBACTERIA IN SOIL AND WATER SAMPLES FROM AROUND KAMPAR, MALAYSIA"** supervised by Dr. Eddy Cheah Seong Guan (Supervisor) from the Department of Biological Science, Faculty of Science.

I hereby give permission to my supervisor to write and prepare manuscripts of these research findings for publishing in any form, if I do not prepare it within six (6) months from this date, provided that my name is included as one of the authors for this article. The arrangement of the name depends on my supervisors.

Yours truly,

_____

(TING CHEE SIONG)

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## LIST OF ABBREVIATIONS & UNITS OF MEASUREMENTS

| | |
|---|---|
| AFB | acid-fast bacilli |
| AIDS | acquired immunodeficiency syndrome |
| BCG | Bacille Calmette-Guérin |
| BLASTn | nucleotide-nucleotide Basic Local Alignment Search Tool |
| $CaCl_2$ | calcium chloride |
| D/W | deionized water |
| $dH_2O$ | distilled water |
| DNA | deoxyribonucleic acid |
| dNTP | deoxyribonucleoside triphosphate |
| EDTA | ethylenediaminetetraacetic acid |
| E-value | expect value |
| GLC | gas-liquid chromatography |
| $H_2O_2$ | hydrogen peroxide |
| HCl | hydrochloric acid |
| HIV | human immunodeficiency virus |
| HPLC | high performance liquid chromatography |
| $K_2HPO_4$ | dipotassium hydrogen phosphate |
| $KH_2PO_4$ | potassium dihydrogen phosphate |
| KTAR | Kolej Tunku Abdul Rahman |
| LB | Luria-Bertani |
| LJ | Löwenstein-Jensen |
| MAC | *Mycobacterium avium* complex |

| | |
|---|---|
| $MgCl_2$ | magnesium chloride |
| $MgSO_4$ | magnesium sulfate |
| Mtb | *Mycobacterium tuberculosis* |
| NaCl | sodium chloride |
| NaOH | sodium hydroxide |
| $Na_3PO_4$ | sodium phosphate |
| NCBI | National Center for Biotechnology Information |
| NTC | no-template control |
| NTM | nontuberculous mycobacteria |
| OADC | oleic acid-albumin-dextrose-catalase |
| PCR | polymerase chain reaction |
| rDNA | ribosomal deoxyribonucleic acid |
| RIDOM | Ribosomal Differentiation of Medical Microorganisms |
| rRNA | ribosomal ribonucleic acid |
| SDS | sodium dodecyl sulfate |
| sp. | species |
| subsp. | subspecies |
| TAE | Tris base-acetate acid-ethylenediaminetetraacetic acid |
| TB | tuberculosis |
| TLC | thin-layer chromatography |
| UTAR | Universiti Tunku Abdul Rahman |
| UV | ultraviolet |
| WHO | World Health Organization |
| ZN | Ziehl-Neelsen |

| | |
|---|---|
| × *g* | times gravity |
| °C | degree Celsius |
| µg | microgram |
| µl | microliter |
| µm | micrometer |
| µM | micromolar |
| bp | base pair |
| cm | centimeter |
| g | gram |
| h | hour |
| kDa | kilodalton |
| min | minute |
| ml | milliliter |
| mm | millimeter |
| mM | millimolar |
| ng | nanogram |
| nM | nanomolar |
| s | second |
| U | unit |
| V | volt |
| v/v | volume per volume |
| W | watt |
| w/v | weight per volume |

# CHAPTER 1

## INTRODUCTION

*Mycobacterium* is a genus of Actinobacteria that is dominated by slow-growing bacteria in which their doubling times are longer compared to those of other bacteria. The minimal requirements for a species to be characterized as *Mycobacterium* are i) acid-fastness, ii) the presence of mycolic acids, which are 60-90 carbons in length, in their cell envelope, and iii) a guanine + cytosine content of 61-71% in their genomes (Shinnick & Good, 1994).

Traditionally, mycobacterial species can be divided into two main groups based on their growth rates. The fast growers take less than seven days to form visible colonies on solid media whereas the slow growers require more than seven days for this (Stadthagen-Gomez *et al*., 2008). In general, slow growing mycobacteria are usually pathogenic to humans and animals, while many rapid growers are considered nonpathogenic (Shinnick & Good, 1994). Mycobacteria can also be grouped according to their ability to produce pigmented colonies: photochromogens (Runyon group I), scotochromogens (Runyon group II), or nonphotochromogens (Runyon group III). Although classifications in these ways have no formal taxonomic standing, they are useful clinically and provide better strategy for identification.

Some mycobacterial species are capable of causing severe diseases. Two major pathogenic species are *Mycobacterium tuberculosis* (Mtb) and *M. leprae*, causative agents for tuberculosis (TB) and leprosy or Hansen' disease, respectively. Leprosy is gradually becoming less important, but TB is currently a global health problem; 8.8 million people fell ill with TB and 1.4 million died from the disease in 2010 (WHO, 2012). Apart from Mtb and *M. leprae*, the genus *Mycobacterium* also consists of nontuberculous mycobacterial (NTM) species. NTM are sometimes referred to as atypical mycobacteria, mycobacteria other than tuberculosis, or environmental mycobacteria in the literature. They are distinguished from the members of the Mtb complex (and *M. leprae*) by the fact that they are not obligate pathogens but are true inhabitants of the environment (Primm, Lucero & Falkinham, 2004).

NTM are opportunistic pathogens that can cause infections in immunocompromised hosts (Kik, Houwers & Dinkla, 2010). They have been reported to cause localized or disseminated disease, depending on local predisposition and/or degree of immune deficit. In leukemia and transplant patients, NTM may cause localized pulmonary disease, adenitis, soft tissue infections, infections of joints or bones, bursae, skin ulcers, and generalized disease. In HIV patients, the clinical manifestations may range from localized to disseminated disease (Katoch, 2004). However, there is no evidence of animal-to-human or human-to-human transmission of NTM (Griffith *et al*., 2007). Hence, environmental exposure is highly likely the source of NTM infections.

Other than causing human infections, NTM can also have negative impacts on the agricultural and farming sectors, which lead to considerable economic losses. Three NTM species have been recognized as potential fish pathogens, namely *M. chelonae*, *M. fortuitum*, and *M. marinum* (Bruno *et al*., 1998). Mycobacteriosis (piscine TB) has been reported to affect a variety of freshwater and marine fish species (Santos, Vale, Sousa & Silva, 2002). *Mycobacterium avium* subsp. *paratuberculosis* can cause Johne's disease in cattle, resulting in decreased milk production and reduced fertility in infected animals (Moghadam, Sarv, Moosakhani & Badiie, 2010).

NTM can be found as saprophytes, commensals, and symbionts in the environment, in which several species may be beneficial (Primm *et al*., 2004). Radhakrishnan, Kumar, and Selvakumar (2010) reported that some NTM species can be used for bioremediation, such as biodegradation of polycyclic aromatic hydrocarbons, crude oils, and other recalcitrant molecules in oil-contaminated sites.

With regard to TB prevention, prior exposure to environmental mycobacteria can affect the efficacy of BCG (Bacille Calmette-Guérin) vaccination. Exposure of mice to *M. avium*, *M. scrofulaceum*, and *M. vaccae*, has been shown to block the replication of the vaccine strain, thereby preventing vaccine protection against Mtb (Brandt *et al*., 2002). Exposure to environmental mycobacteria can also affect the specificity of the tuberculin skin test, which is used to screen individuals for latent TB infection.

In this project, isolation and identification of NTM in soil and water samples from around Kampar were carried out. As we know, environmental samples are rich in a variety of microorganisms, many of which can grow rapidly and outcompete the slow-growing mycobacteria *in vitro*. This challenge was addressed by subjecting the samples to a published decontamination treatment prior to inoculation onto Löwenstein-Jensen media; mycobacteria are relatively resistant to the chemical agents used due to their thick cell envelope (Parashar *et al*., 2004). Culture isolates were confirmed to be those of mycobacteria based on Ziehl-Neelsen (ZN) acid-fast staining and examination of colony morphology and growth rates. They were then identified to the species level based on their biochemical characteristics and 16S ribosomal DNA (rDNA) sequences. The health impacts of the isolated NTM species on human and animal hosts were briefly assessed.

The aims and objectives of this project were:

I. to isolate and identify NTM species in soil and water samples;
II. to study the distribution of NTM in different environmental reservoirs and sampling locations;
III. to assess the health impacts of the isolated NTM species on human and animal hosts.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Mycobacteria in General

*Mycobacterium* is a genus within the phylum Actinobacteria, assigned to the family *Mycobacteriaceae*. The prefix "myco-" means both fungus and wax; the former refers to the mold-like growth of mycobacteria on the surface of liquid media, while the latter refers to the waxy compounds in their cell wall. Mycobacteria can be subdivided into three major groups: Mtb complex, *M. leprae*, and NTM. The Mtb complex is a group of closely related mycobacterial species that can cause TB in human. The members of this group include Mtb, *M. bovis*, *M. africanum*, *M. microti*, *M. canettii*, *M. pinnipedii*, *M. caprae*, and *M. mungi*. *M. leprae*, the causative agent for leprosy, is an obligate intracellular parasite that lacks many necessary genes for independent survival; therefore, researchers face a problem to grow it in artificial media (Barker, 2006).

Mycobacterial cells are straight or slightly curved rods measuring 0.2-0.6 by 1-10 µm in size. They are aerobic and non-motile bacilli, except for *M. marinum*, which exhibits motility in macrophages (Stamm *et al*., 2003). Mycobacteria cannot form endospores with the exception of old cultures of *M. bovis* BCG and *M. marinum* (Singh, Ghosh, Islam, Dasgupta & Kirsebom, 2010). They are neither true Gram-positive nor Gram-negative bacteria but are characteristically acid-fast. Although they lack of an outer membrane, their cell wall is hydrophobic, waxy, and thicker than that of many other bacteria, owing

to the presence of large amounts of mycolic acids. These are branch chained fatty acids that are 60-90 carbons in length. The mycolic acids and other glycolipids impede the entry of hydrophilic nutrients and chemicals, causing mycobacteria to grow slowly and to be more resistant to chemical agents than most other bacteria, respectively (Liu, 2011).

Mycobacteria have relatively simple nutritional requirements. They can adapt readily to growth on simple mineral salt medium, using ammonia as the nitrogen source and glycerol as the carbon source (Falkinham III, 2002). Mycobacteria species that form visible colonies within seven days on solid medium at optimal temperature are called rapid growers, while those requiring longer duration of incubation are called slow growers. However, the growth of rapid-growing species is still much slower than that for most bacteria. Apart from impermeability of their cell wall to certain nutrients, slow growth among mycobacteria is also possibly due to the energy cost for the synthesis of long-chain mycolic acids and the presence of very low numbers of 16S ribosomal RNA cistrons in their genomes; the latter limits the rate of protein synthesis (Primm *et al*., 2004). Optimal growth temperatures vary widely among different species and generally range from 25 °C to 50 °C. Optimal pH values for growth also vary among different species; those for slow growers are generally between 5.8 and 6.5, while those for rapid growers are between 7 and 7.4 (Portaels & Pattyn, 1982).

## 2.2 Nontuberculous Mycobacteria

### 2.2.1 Nontuberculous Mycobacteria in General

NTM are mycobacterial species that may cause human disease but not TB. They are true inhabitants of the environment and can be isolated from a variety of environmental reservoirs such as natural waters, drinking waters, soils, biofilms, aerosols, and dusts. They have even been isolated from cigarettes (Falkinham III, 2002). NTM include both slow- and rapid-growing species. *M. avium* and *M. intracellulare*, collectively known as the *M. avium* complex (MAC), are the most important slow growers as they are commonly recovered from immunocompromised patients.

### 2.2.2 Diseases in Human Hosts

NTM have been reported to cause localized or disseminated disease, depending on local predisposition and/or degree of immune deficit (Katoch, 2004). It is a common observation that NTM cause disease in individuals who offer some opportunity due to altered local or systemic immunity. MAC is primarily a pulmonary pathogen that affects individuals who are suffering from acquired immunodeficiency syndrome (AIDS) and hairy cell leukemia, and those on immunosuppressive chemotherapy. According to Koirala (2012), MAC lung disease does not occur in immunocompetent hosts. However, those with underlying lung disease may develop progressive MAC lung infection. MAC can also cause the Lacy Windermere syndrome, a disease associated with pulmonary infection and bronchiectasis in elderly women without pre-existing lung disease (Koirala, 2012). NTM like MAC and *M. scrofulaceum* can also

cause lymphadenitis in children. Other NTM such as *M. szulgai*, *M. marinum*, *M. ulcerans*, and *M. vaccae* have also been reported to cause skin infections.

### 2.2.3 Diseases in Animal Hosts

Besides humans, animals are susceptible to mycobacterial infections as well. One example is the Johne's disease in ruminant species that is caused by *M. avium* subsp. *paratuberculosis*. This disease primarily affects the digestive system of ruminants and manifests as diarrhea and wasting, resulting in weight loss and eventual death of the animals (Meadows, 2004). Another common NTM disease in animals is mycobacteriosis or fish TB, which can be due to *M. fortuitum, M. flavescens, M. chelonae, M. gordonae, M. terrae, M. triviale, M. diernhoferi, M. celatum, M. kansasii, M. intracellulare*, or *M. marinum* (Moseley, 2011). The infected fish is often emaciated and lethargic with abnormal growth in the liver and spleen.

### 2.2.4 Impact on the Food Industry

NTM can affect the food industry due to the diseases they cause in food animals, which can lead to considerable economic losses. In cattle, Johne's disease can decrease milk production and reduce fertility (Moghadam *et al.*, 2010). More importantly, consumption of milk products contaminated by *M. paratuberculosis* increases the risk of Crohn's disease in consumers. All fish are susceptible to mycobacteriosis. This disease has been reported in a broad range of fish species from fresh water, brackish water, and marine environments. In fresh water systems, striped bass and their hybrids seem especially susceptible. In mariculture, mycobacteriosis has been documented in

Florida pompano, sea bass, and turbot (Francis-Floyd, 2011). This can reduce fish yield and subsequently affect food supply.

### 2.2.5 Bioremediation

*M. frederiksbergense, M. gilvum, M. austroafricanum, M. vanbaalenii, M. holderi, M. flavescens, M. anthracenicum,* and *M. chelonae* have been reported to be able to degrade polycyclic aromatic hydrocarbons, crude oils, and chemical dyes and other recalcitrant molecules in polluted environments (Radhakrishnan *et al*., 2010). These mycobacteria may boost the bioavailability of polycyclic aromatic hydrocarbons by excreting biosurfactants, by production of extracellular polymeric substances, or by forming biofilms (Johnsen & Karlson, 2003). Another NTM species, *M. vaccae*, has been reported to be able to degrade alkanes, acetone, benzene, dioxane, styrene, and trichloroethylene and to degrade and transform sterols (Falkinham III, 1996). The latter ability has also been documented for *M. phlei* (Jabbouri *et al.*, 1991).

## 2.3 Isolation of Nontuberculous Mycobacteria from the Environment

### 2.3.1 Previous Studies in Different Geographical Regions

NTM are ubiquitously distributed in the environment; they are found in fresh and salt water, soil, food, and dust. However, most studies focused on isolation of NTM in soil and water samples. Jones and Jenkins (1965) demonstrated the presence of large populations of fast-growing NTM species in soil environments. According to Tsukamura (1984), *M. fortuitum* is probably the most frequently occurring NTM species in the environment and is often the major species (40-80%) isolated from soil samples. Apart from *M. fortuitum*,

other rapid-growing NTM species (*M. aurum*, *M. smegmatis*, and *M. agri*) and the slow-growing *M. nonchromogenicum* complex (*M. nonchromogenicum*, *M. terrae*, and *M. triviale*) are also commonly isolated from soil samples.

Mycobacteria are susceptible to short-wavelength ultraviolet (UV) radiation (Falkinham III, 2009) and therefore soil samples collected must not be exposed to too much sunlight in order to recover them successfully. The recommendation is for soil samples to be collected from the depth of 3 cm (Parashar *et al*., 2004; Rahbar, Lamei, Babazadeh & Yavari, 2010).

*M. gordonae* is the NTM species most often isolated from tap water samples of various origins (Collins, Grange & Yates, 1984). Strains of *M. terrae*, *M. phlei*, and *M. fortuitum* dominate various non-marine aquatic environments (Hartmans, Bont & Stackebrandt, 2006) while members of the *M. avium-intracellulare-scrofulaceum* complex are able to be isolated from variety of water sources including marine water (Tuffley & Holbeche, 1980). Apart from NTM, members of the Mtb complex such as Mtb and *M. bovis* have also been isolated from the environment (Kazda, 1983).

### 2.3.2 Processing of Samples

Mycobacteria are difficult to isolate from environmental samples as they grow relatively slow and are therefore easily outcompeted by other faster-growing bacteria that are present in great abundance. In this case, prior decontamination of samples is of utmost importance in facilitating successful isolation of mycobacteria. A nonstringent decontamination method used could result in

overgrowth of contaminants, while method that is too stringent could result in the loss of mycobacteria (Kamala, Paramasivan, Herbert, Venkatesan & Prabhakar, 1993a). Other than that, all mycobacterial species are not equally resistant to a particular decontamination procedure (Parashar *et al*., 2004). Therefore, the method chosen for decontamination is critical to the isolation process. Most published methods make use of chemical agents for decontamination purpose, taking advantage of the high resistance of mycobacteria to them (Table 2.1).

**Table 2.1: Decontamination methods developed and used in previous studies**

| Method | Chemical(s) used[†] | Reference |
|---|---|---|
| Falkinham's method | 1% NaOH, 2% NaOH, and 4% NaOH | Brooks, Parker, Gruft & Falkinham III, 1984 |
| Gangadharam's method | 1% cetrimide | Joseph, Nair & Gangadharam, 1969 |
| Corper and Stoner's method | 10% $Na_3PO_4$ | Corper and Stoner, 1946 |

[†] Concentrations in w/v; NaOH, sodium hydroxide; $Na_3PO_4$, sodium phosphate.

Besides the methods listed in Table 2.1, combination of two or more chemicals, for example, 3% w/v sodium dodecyl sulfate (SDS) + 1% w/v NaOH (Engbaek, Vergmann & Bentzon, 1967; Engel, Berwald & Havelaar, 1980) or 3% w/v SDS + 4% w/v NaOH + 2% w/v cetrimide (Parashar *et al.*, 2004) were shown to have better decontamination power in highly contaminated samples. Although several comparisons of different decontamination methods had been conducted to determine an optimal method, there is no clear consensus to this as the effectiveness of a decontamination method depends on the type of samples tested and the degree of tolerance of the mycobacterial species present.

## 2.4 Laboratory Identification of Mycobacteria

### 2.4.1 Culture

Media used to cultivate mycobacteria have been mainly developed for the isolation of species of medical importance, especially Mtb. Robert Koch first succeeded in isolating mycobacteria using coagulated bovine serum. After that, many types of culture media were formulated in the form of egg-based, agar-based, or liquid media. Both liquid and solid media can be made selective for mycobacteria via addition of antibiotics or chemical agents (Parish & Stoker, 1998).

The first egg-based medium was developed by Dorset in 1903, followed by a variety of other recipes like Löwenstein-Jensen (LJ), American Trudeau Society, Ogawa, and Petragnani. The latter four each contains different concentration of malachite green, an inhibitory agent used to control non-mycobacterial contaminants. LJ medium is the most commonly used medium for the culture of mycobacteria, which is also recommended by the World Health Organization (WHO, 1998).

The most widely used agar-based medium is Middlebrook 7H10. The composition of Middlebrook 7H11 agar is similar to that for Middlebrook 7H10, but with the presence of enzymatic digest of casein and higher concentration of malachite green in the former. Blood agar has also been used to culture Mtb and was reported to be at least as efficient as LJ medium (Drancourt, Carrieri, Gévaudan & Raoult, 2003). It has the advantage of promoting rapid growth of mycobacteria, which in turn allows earlier

observation of growing colonies (Drancourt *at al*,. 2003; Mathur, Gaur, Sharma & Solanki, 2009).

Apart from conventional methods, fully-automated culture systems are also available for isolation of mycobacteria. They have been reported to be more rapid and sensitive for the detection of mycobacterial growth, when compared to traditional methods (Kampen, Anthony & Klatser, 2010). One example is the MB/BacT System, a fully automated, nonradiometric system designed for the recovery of mycobacteria (Piersimoni *et al*., 2001).

### 2.4.2 Acid-fast Staining

Mycobacteria contain high proportions of hydrophobic waxy lipids, especially mycolic acids, in their cell wall that render them impermeable to many stains, including the Gram stain. However, this unique characteristic enables mycobacterial cells to resist decolorization by acid-alcohol following a specific staining procedure; therefore they are referred to as acid-fast bacilli (AFB).

ZN, Kinyoun, and auramine-rhodamine fluorochrome staining are the three common acid-fast staining methods used today. ZN and Kinyoun staining are also known as hot and cold staining, respectively (Hussey & Zayaitz, 2008). Both these methods are basically similar, but are slightly different in the way that ZN staining uses heat to facilitate penetration of carbol fuchsin into cell wall while Kinyoun staining relies on increased concentration of basic fuchsin and phenol to achieve this. The identification of mycobacteria with auramine-rhodamine staining is based on the affinity of mycolic acids for the

fluorochrome dyes. It is more sensitive than the ZN staining but is less specific for acid-fast organisms; therefore it is usually utilized as screening tool. One of the limitations of acid-fast staining is that it is not specific for detection of mycobacteria; it can also stain other actinobacteria such as *Norcadia* and *Rhodococcus*. Furthermore, smear-positive and culture-negative cases are possible due to the inability of acid-fast staining to differentiate between live and dead mycobacterial cells.

### 2.4.3 Biochemical Tests

Biochemical tests are one of the conventional methods for identification of mycobacterial species based on their phenotypic characteristics. A set of tests is performed for this purpose and their principles are usually based on the presence of certain enzymes in mycobacteria or their ability to grow in certain selective media (Table 2.2).

Although biochemical tests can be performed easily in the laboratory, they impose a number of limitations. They can be time-consuming due to long incubation time. In addition, interspecies homogeneity, intraspecies variability, and the existence of undescribed species can lead to phenotypic misidentification.

**Table 2.2: Biochemical tests used for identification of mycobacteria**

| Biochemical test | Purpose |
|---|---|
| Arylsulfatase test | To test for the presence of arysulfatase, which hydrolyzes bond between the sulfate group and aromatic ring of tripotassium phenolphthalein disulfate |
| Catalase tests: | |
| Semiquantitative test | To test for the presence of catalase and the amount of the enzyme produced |
| Heat tolerance test | To test for the heat stability of catalase at 68 °C |
| Iron uptake | To test for the ability of rapid growers to convert ferric ammonium citrate to iron oxide |
| Niacin accumulation | To test whether niacin is excreted out from mycobacterial cells |
| Nitrate reduction | To test for the presence of nitrate reductase, which catalyzes the reduction of nitrate to nitrite |
| Pyrazinamide sensitivity | To test for the presence of pyrazinamidase, which hydrolyzes pyrazinamide to ammonia and pyrazinoid acid |
| Sodium chloride (NaCl) tolerance | To test for the ability of mycobacteria to grow in medium containing 5% w/v NaCl |
| Inhibition by thiophene-2-carboxylic acid hydrazide | To test for the resistance of mycobacteria to thiophen-2-carboxylic acid hydrazide at concentrations of 1-5 µg/ml |
| Tellurite reduction | To test for the ability of mycobacteria to reduce tellurite to metallic tellurium and to assess their rate of reduction |
| Tween 80 hydrolysis | To test for the presence of lipase, which hydrolyzes the detergent Tween 80 to oleic acid and polyoxyethylene sorbitol |
| Urease test | To test for the presence of urease, which hydrolyzes urea to ammonia and carbon dioxide |

Adapted from "Handbook of Clinical Microbiology Procedures" (Garcia & Isenberg, 2010)

### 2.4.4 Genotypic Identification

Over the last 20 years, the development of molecular techniques has had a major impact on the diagnosis of mycobacterial infections**.** Several molecular methods have been developed for mycobacterial identification, including the use of specific DNA probes, conventional polymerase chain reaction (PCR), real-time PCR, PCR hybridization with species-specific probes, and nucleic acid sequence analysis.

The AccuProbe system (Gen Probe) involves the use of species-specific DNA probes for mycobacterial identification. It is a nucleic acid hybridization assay that is based on the ability of complementary nucleic acid strands to come together to form stable double-stranded complexes under appropriate conditions. The accuracy of identification with this method has been reported to be nearly 100% (Woods, 2002). However, probes specific to only four species and two complexes have been developed so far; these target *M. avium*, *M. intracellulare*, *M. kansasii*, *M. gordonae*, Mtb complex, and *M. avium-intracellulare* complex (McNabb, Adie, Rodrigues, Black & Isaac-Renton, 2006). Furthermore, the Mtb complex identification test does not differentiate the members within the complex. Other examples of probe hybridization assays are the GenoType Mycobacterium test (Hain Lifesciences) and the INNO-LiPA MYCOBACTERIA V2 assay (Innogenetics).

Conventional PCR and real-time PCR are basically similar; the difference is that the latter enables simultaneous quantification of target DNA molecules during PCR amplification based on the use of fluorescence detection chemistry.

The targets can be species-specific genes or housekeeping genes; PCR assays for the latter are useful for identification of unknown mycobacterial species in combination with downstream DNA sequencing of amplicons. Examples of housekeeping genes that have been targeted include the 16S rRNA gene (Cheah, 2010), *hsp65* (Ringuet *et al*., 1999), 32-kDa protein gene (Soini, Bottger & Viljanen, 1994), *rpoB* (Adekambi, Colson & Drancourt, 2003), and *recA* (Blackwood *et al.*, 2000). Sequencing of the 16S rDNA has emerged as the single best method to identify bacteria rapidly (Drancourt *et al.*, 2000). The 16S rDNA for almost all mycobacterial species have been sequenced and analyzed by various laboratories (Han, Pham, Tarrand, Sood & Luthra, 2002). The availability of these sequences through public and private databases, such as the GenBank and the Ribosomal Differentiation of Medical Microorganisms (RIDOM) respectively, enables the identification of mycobacterial species by performing alignment search.

### 2.4.5 Other Methods

There are also other methods for the identification of mycobacteria. Thin-layer chromatography (TLC), gas-liquid chromatography (GLC), and high performance liquid chromatography (HPLC) all perform mycobacterial identification through analysis of mycolic acids in their cell wall. TLC is good for resolving single mycolic acid types in which results are presented as spots on the chromatographic plate (Brennan, Heifets & Ullom, 1982). GLC is used for the detection of characteristic cleavage products of mycobacterial alcohols and long-chain fatty acids (Guerrant, Lambert & Moss, 1981). Combination of

GLC with mass spectrometry has also been used to facilitate the determination of size of mycolic acids (Kaneda *et al.*, 1986).

The phage amplification assay is another rapid non-molecular-based method for the identification of mycobacteria, based on specific infection by mycobacteriophages and formation of visible plaques. The method is quantitative in the sense that the number of plaques detected correspond to the number of viable mycobacterial cells present in the test sample. Studies show that different types of mycobacteriophages infect different types of mycobacteria. Mycobacteriophage Bxb1 infects only fast-growing mycobacteria but not slow growers. However, others such as D29, TM4, and L5 infect both fast- and slow-growing mycobacterial species (Peng, Chen, Luo & Wang, 2006). Generally, results can be obtained within two days with this method, compared to several weeks for culture-based methods.

Serological-based methods for detection of mycobacteria are also available. Different mycobacterial antigens have been targeted in serological tests, including liporabinomannan, purified protein derivatives, 38-kDa glycoprotein, heat shock proteins, phenolic glycolipids, A60 antigen complex, and excretory-secretory antigens. A commercial serological test, the BrockTB Stat-Pak system (Chembio Diagnostic System), is available for the diagnosis of mycobacterial infections in wildlife (Lyashchenko *et al.*, 2008). Serological tests have relatively lower sensitivity of detection but they are simple, rapid, inexpensive, and suitable for field application.

# CHAPTER 3

# METHODOLOGY

## 3.1 Experimental Design

The overall experimental design for this project is summarized in Figure 3.1.

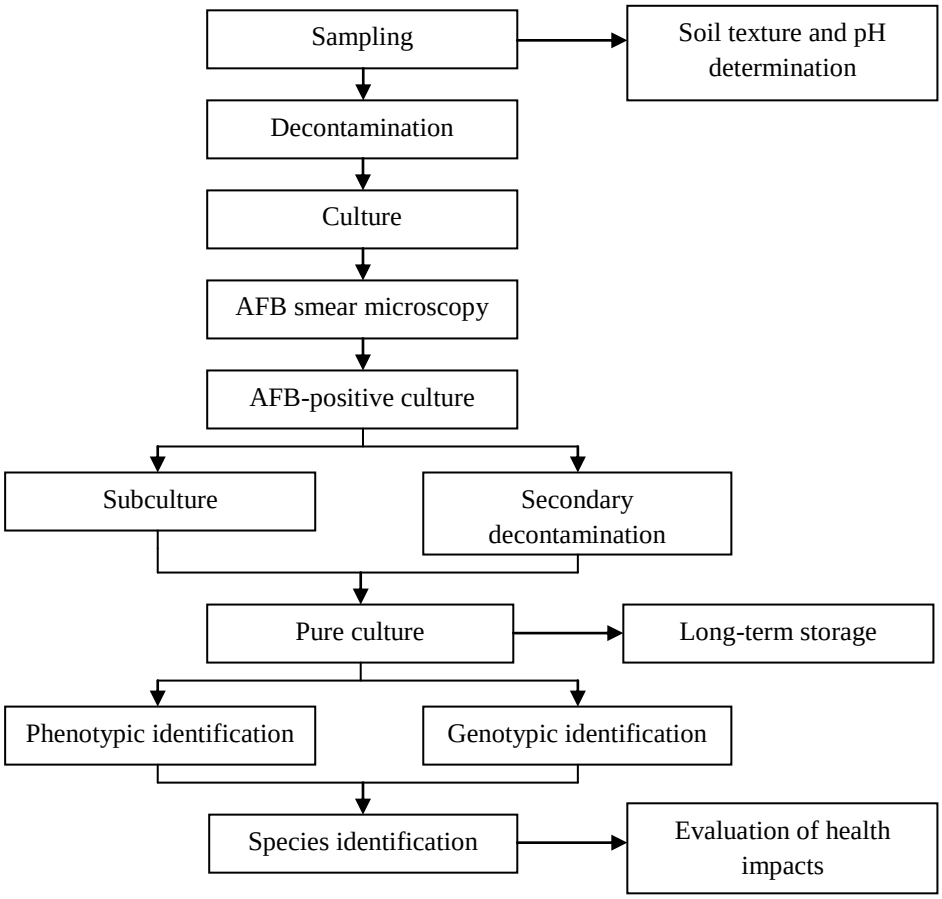


**Figure 3.1: Overview of the experimental design for the project**

## 3.2 Apparatus and Consumables

The apparatus and consumables (and their respective manufacturers) used in this project are listed in Appendix A.

### 3.3 Preparation of Culture Media

All media were sterilized by autoclaving at 121 ºC for 2 h unless otherwise stated. All filter sterilization procedures were performed with 0.2-μm membrane filters.

#### 3.3.1 Löwenstein-Jensen Media

An amount of 9.325 g LJ medium base and 3 ml of glycerol were dissolved in 147 ml of distilled water ($dH_2O$). The mixture was stirred and boiled for 1 min. LJ bases with salt and antibiotic were prepared as described above, with the addition of 5% w/v NaCl and 50 U/ml penicillin G, respectively. The LJ base was then autoclaved.

The following steps were performed aseptically in the Class 2 microbiological safety cabinet. A total volume of 250 ml of egg was homogenized in a sterile beaker, and then the homogenate filtered through gauze and mixed with the LJ base. Following thorough mixing, the mixture was distributed into sterile 30-ml universal bottles or test tubes in 10-ml aliquots. Then, the media were inspissated at 85 ºC for 2-3 h in slanted position for those in universal bottles and upright position for those in test tubes. LJ media were then incubated at 37 ºC overnight to check for contamination.

#### 3.3.2 Luria-Bertani Agar

An amount of 18.5 g of LB agar powder was dissolved in $dH_2O$ to a final volume of 500 ml, and then boiled for 5 min to dissolve the agar powder completely before autoclaving.

### 3.3.3 Middlebrook 7H9 Broth and Agar

Middlebrook 7H9 broth was prepared by mixing 0.94 g of Middlebrook 7H9 broth powder and 0.5 g of glycerol with $dH_2O$ to a final volume of 180 ml. Middlebrook 7H9 agar was prepared as described above, with the addition of 3 g of agar-agar powder. The agar was boiled for 5 min before autoclaving.

### 3.3.4 Tween 80 Substrate Solution

Tween 80 substrate solution was prepared by mixing 0.5 ml of Tween 80 and 2 ml of 1% w/v phenol red in 67 mM phosphate buffer to a final volume of 100 ml. The mixture was then filter sterilized.

### 3.3.5 Urea Broth

Urea broth was prepared by mixing 1 g of peptone, 1 g of dextrose, 5 g of NaCl, 0.4 g of $KH_2PO_4$, 20 g of urea, 1 ml of 1% v/v phenol red and 0.1 ml of Tween 80 in $dH_2O$ to a final volume of 1000 ml. The mixture was then filter sterilized.

## 3.4 Preparation of Reagents

All reagents were sterilized by autoclaving at 121 ºC for 2 h unless otherwise stated. All filter sterilization procedures were performed with 0.2-μm membrane filters.

### 3.4.1 Acid-alcohol

Acid-alcohol was prepared by mixing 3 ml of HCl with 95% v/v ethanol to a final volume of 100 ml.

### 3.4.2 Calcium Chloride Solution, 0.1 M

An amount of 2.77 g of $CaCl_2$ was dissolved in 25 ml of $dH_2O$ to make a 0.1 M solution. The solution was then filter sterilized.

### 3.4.3 Carbol Fuchsin Solution

An amount of 0.3 g of basic fuchsin was dissolved in 10 ml of 95% v/v ethanol and 5 ml of heat-melted phenol crystals was dissolved in 95 ml of $dH_2O$. Both solutions were mixed and left to stand for two days. The mixture was filtered with a filter paper before use.

### 3.4.4 Catalase Reagent

Catalase reagent was prepared by mixing 30% v/v $H_2O_2$ and 10% w/v Tween 80 in 1:1 ratio.

### 3.4.5 Cetrimide Solution, 1% w/v and 2% w/v

Cetrimide solution with concentrations of 1% w/v and 2% w/v were prepared by mixing 4 g and 8 g of cetrimide, respectively, with $dH_2O$ to a final volume of 400 ml. The solution was then filter sterilized.

### 3.4.6 Glycerol Solution, 65% v/v

Glycerol solution was prepared by mixing 162.5 g of glycerol, 20 ml of 1 M $MgSO_4$, and 5 ml of 1 M Tris-HCl (pH 8) with $dH_2O$ to a final volume of 200 ml.

### 3.4.7 Methylene Blue

The stock solution was prepared by mixing 0.7 g of methylene blue with $dH_2O$ to a final volume of 50 ml. The solution was filtered using filter paper. The filtered stock solution was diluted 10 times with $dH_2O$ before use.

### 3.4.8 Phosphate Buffer, 67 mM, pH 6.8

Phosphate buffer was prepared by mixing 0.2 M $KH_2PO_4$ and 0.2 M $K_2HPO_4$ to final concentrations of 34 mM and 33 mM, respectively.

### 3.4.9 SDS Solution, 3% w/v

An amount of 24 g of SDS was dissolved in $dH_2O$ to a final volume of 400 ml to prepare a 6% w/v solution. The solution was filter sterilized and diluted to 3% w/v with $dH_2O$ before use.

### 3.4.10 Sodium Hydroxide Solution, 4% w/v

An amount of 16 g of NaOH pellets was dissolved in $dH_2O$ to a final volume of 400 ml to prepare a 4% w/v solution.

### 3.4.11 TAE Buffer

The stock solution was prepared by mixing 242 g of Tris base, 37.2 g of EDTA, and 57.1 ml of glacial acetic acid with $dH_2O$ to a final volume of 1000 ml. The stock solution was diluted 50 times with $dH_2O$ before use.

### 3.4.12 Tween 80, 10% w/v

Ten grams of Tween 80 stock solution was dissolved in $dH_2O$ to a final volume of 100 ml. The solution was warmed at 40 ºC until Tween 80 completely dissolved. The solution was then filter sterilized and stored at 4 °C away from strong light.

## 3.5 Sampling

### 3.5.1 Collection of samples

Three bovid-contacted grounds around Kampar were selected for soil sampling; one of them is a cow farm in Taman Bandar Baru Selatan, while the other two are buffalo-feeding grounds in Eastlake and near to Kolej Tunku Abdul Rahman (KTAR), respectively. Soil samples were collected from three random sites for each sampling location; the sites were more than 5 m apart from each other. Approximately 100 g of soil was collected from a depth of 3 cm from each site using a shovel.

Three water reservoirs around UTAR Perak Campus were selected for water sampling; one of them is the river next to Block F, while the other two are ex-tin-mining lakes near to the Sport Complex and Block C, respectively. Similarly, water samples were collected from three random sites for each sampling location. Approximately 50 ml of water was collected in a 500-ml Schott Duran bottle from each site.

The visual appearance of both soil and water samples was assessed. Sample analyses were performed on days of sampling if possible. If not, they would be

stored under recommended conditions, which are refrigeration for water samples and ambient storage for soil samples (Kamala, Paramasivan, Herbert, Venkatesan & Prabhakar, 1993b).

### 3.5.2 Soil pH Determination

Twenty grams of soil sample was transferred to a 50-ml centrifuge tube. Distilled water was added to the sample in a 1:1 ratio. The sample was shaken and then left to stand for 30 min; this step was repeated for three times. The pH of the soil sample was measured using a pH meter. Triplicate measurements were made for each sample.

## 3.6 Chemical Decontamination

### 3.6.1 Primary Decontamination

The decontamination of both soil and water samples were carried out as described by Parashar *et al.* (2004).

For soil analysis, 5 g of soil was suspended in 20 ml of deionized water (D/W) in a 50-ml centrifuge tube. The suspension was shaken gently for 60 s and then centrifuged at 600 × *g* for 5 min. Then, 10 ml of the resulting supernatant was transferred to a new tube and centrifuged at 8000 × *g* for 15 min. For water analysis, 50 ml of collected water was transferred into a 50-ml centrifuge tube and then centrifuged at 8000 × *g* for 15 min.

The procedures for both soil and water samples were identical from this point onwards. Following centrifugation, the resulting pellet was resuspended in 20

ml of 3% w/v SDS + 4% w/v NaOH. The suspension was then divided into two parts, A and B. Part A was incubated at room temperature for 15 min and part B incubated for 30 min to obtain the growth of rapid- and slow-growing mycobacteria, respectively. After incubation, the suspension was centrifuged at 8000 × *g* for 15 min and the resulting pellet resuspended in 20 ml of 2% w/v cetrimide. The pellet from part A was incubated for 5 min at room temperature and that from part B incubated for 15 min. The suspension was subsequently centrifuged at 8000 × *g* for 15 min and the resulting pellet resuspended in 20 ml of D/W. The suspension was centrifuged as before and the resulting pellet resuspended in 0.5 ml of D/W for inoculation onto LJ slants. All centrifugation steps were performed at room temperature.

### 3.6.2 Secondary Decontamination

#### 3.6.2.1 Decontamination with Cetrimide

A loopful of colonies from a mixed culture was treated with 5 ml of cetrimide solution at room temperature. Different concentrations of cetrimide (1% and 2% w/v) and durations of treatment were evaluated. Following treatment, the suspension was centrifuged at 8000 × *g* for 15 min and the resulting pellet resuspended in 5 ml of D/W. The suspension was then centrifuged as before and the resulting pellet resuspended in 0.5 ml of D/W for inoculation onto LJ slants.

### 3.6.2.2 Decontamination with Sodium Hydroxide

A loopful of colonies from a mixed culture was emulsified in 0.5 ml of sterile D/W and then treated with 0.5 ml of NaOH solution at room temperature. Different concentrations of NaOH (2% and 4% w/v) and durations of treatment (15 and 30 min) were evaluated. Following treatment, 1 ml and 2 ml of 14% w/v $KH_2PO_4$ were added to the 2% and 4% w/v NaOH suspensions, respectively. Each suspension was then centrifuged at 2000 × *g* for 15 min and the resulting pellet resuspended in 2 ml of D/W for inoculation onto LJ slants.

## 3.7 Culture

For every decontamination treatment, 0.1 ml of suspension was inoculated onto LJ slants in duplicate. One slant was incubated at 37 ºC and the other at 30 ºC. Bacterial growth on and physical appearance of the LJ slants were observed on a daily basis for up to two months. The colony morphology and growth rates of mycobacterial isolates were recorded. For an AFB-positive culture with little or no contamination, a well-isolated colony was picked and then streaked onto another LJ slant. For AFB-positive cultures that were heavily contaminated, secondary decontamination was carried out as described in Section 3.6.2. After decontamination, 0.1 ml of suspension for each culture was inoculated onto a LJ slant.

### 3.7.1 Isolation of Contaminant Organisms

A well-isolated contaminant colony from a primary culture was picked and streaked onto a LB plate. The plate was then incubated at the isolation temperature of the primary culture.

### 3.8 Bacterial Staining

#### 3.8.1 Ziehl-Neelsen Acid-fast Staining

A smear was prepared by emulsifying a colony in a drop of $dH_2O$ or spreading a loopful of liquid culture on a microscope slide. The smear was left to air dry and subsequently heat-fixed. Then, the slide was flooded with carbol fuchsin solution. The slide was heated until steam appeared and then left to stand for 5 min. After that, the slide was rinsed with tap water and then flooded with acid-alcohol. The slide was rinsed as before again and then flooded with methylene blue counterstain. After 1 min, the slide was rinsed with tap water and then blotted dry. Finally, the slide was examined under oil immersion at 1000× magnification.

#### 3.8.2 Gram Staining

A smear was prepared by emulsifying a colony in a drop of $dH_2O$ on a microscope slide. The smear was left to air dry and then heat-fixed. The slide was first flooded with crystal violet for 1 min and then rinsed with tap water. Next, the slide was flooded with iodine for 30 s and then rinsed as before. After that, the slide was flooded with 95% v/v ethanol for a few seconds and then rinsed with tap water. The slide was then flooded with the counterstain safranin for 30 s and then rinsed as before. The slide was blotted dry and then examined under oil immersion at 1000× magnification.

### 3.9 Phenotypic Identification

#### 3.9.1 Pigmentation Test

A loopful of colonies from a pure mycobacterial isolate was emulsified in 0.5 ml of D/W. A volume of 0.1 ml of the suspension was inoculated onto each of three LJ slants. Two LJ slants were wrapped with aluminum foils. All slants were incubated at the initial isolation temperature of the isolate. Once growth was observed on the unwrapped slant, one of the wrapped slants was exposed to light from a 100-W tungsten bulb from a distance of 20 cm for 5 h. The exposed slant was rewrapped and then reincubated for another 24 h. Finally, all aluminum foils were removed and the color of colonies formed was observed and recorded.

#### 3.9.2 Biochemical Tests

Pure mycobacterial isolates were used in all tests. All incubation steps were performed at the respective isolation temperature for each primary isolate, unless otherwise stated. A negative control was performed for each test.

##### 3.9.2.1 Semiquantitative Catalase Test

A loopful of colonies from an isolate was emulsified in 0.2 ml of D/W. A volume of 0.1 ml of the suspension was inoculated onto a LJ butt and then incubated for two weeks. One ml of catalase reagent was added to the LJ butt and left for 5 min at room temperature. The butt was then observed for bubble formation and the height of the bubble column measured and recorded as either above or below 45 mm from the surface of the butt.

#### 3.9.2.2 Thermostable Catalase Test

A loopful of colonies from an isolate was suspended in 0.5 ml of phosphate buffer in a 50-ml centrifuge tube. The resulting suspension was incubated at 68 ºC for 20 min and then cooled to room temperature. Then, 0.5 ml of catalase reagent was added and the tube was observed for bubble formation.

#### 3.9.2.3 Urease Test

One part of urea broth was mixed with nine parts of D/W and then dispensed in 4-ml aliquots in 50-ml centrifuge tubes. A loopful of colonies from an isolate was suspended in the test medium and incubated for up to two weeks. The tube was observed for color change daily. A development of pink color in the test medium would indicate a positive reaction.

#### 3.9.2.4 Salt Tolerance Test

A loopful of colonies from an isolate was suspended in 0.2 ml of D/W. A volume of 0.1 ml of the suspension was inoculated onto a LJ slant containing 5% w/v NaCl and incubated for four weeks. Bacterial growth on the slant was recorded.

#### 3.9.2.5 Tween 80 Hydrolysis Test

A loopful of colonies from an isolate was emulsified in 4 ml of Tween 80 substrate solution in a 50-ml centrifuge tube and incubated up to two weeks. The tube was observed for color change daily. A color change from red to yellow would indicate a positive reaction.

### 3.9.3 Mycobacteriophage Infection

A few loopfuls of colonies from an isolate were emulsified in 4 ml of Middlebrook 7H9 broth in a 50-ml centrifuge tube. The resulting suspension was vortexed in the presence of glass beads for 1 min. Then, 1 ml of the suspension was transferred to 9 ml of Middlebrook 7H9 molten agar (supplemented with 10% v/v OADC and 1 mM $CaCl_2$) in a 50-ml centrifuge tube. The tube was inverted a few times for thorough mixing before its content was poured onto a petri dish. The solidified plate was left to dry under laminar air flow for 30 min. Sextuplicate 20-µl drops of mycobacteriophage D29 suspension were then spotted onto the agar surface and the plate was left to dry as before. The plate was incubated at 37 °C for up to three days and then observed for plaque formation.

## 3.10 Genotypic Identification

### 3.10.1 DNA Release by Boiling

A loopful of colonies from an isolate was suspended in 0.2 ml of D/W. The suspension was boiled at 100 ºC for 30 min and then centrifuged at 16100 × $g$ for 2 min. The resulting supernatant was used as template DNA in PCR assay.

### 3.10.2 16S rDNA PCR Assays

Two different 16S rDNA PCR assays were performed during this project, the *Mycobacterium*-genus specific 16S assay and the universal 16S assay for mycobacterial and contaminant isolates, respectively. For the former, PCRs were carried out in 25-µl volumes containing 5 µl of template DNA, 250 nM of each forward (5'-GAAACTGGGTCTAATACCG-3') and reverse (5'-

ATCTCAGTCCCAGTGTGG-3') *Mycobacterium* genus-specific primers, 1.25 U of DreamTaq Green DNA polymerase, 1x buffer (contains 2 mM $MgCl_2$), and 200 µM dNTPs. PCRs were performed in the thermocycler as follows: 95 °C for 3 min to activate the *Taq* polymerase, followed by 40 cycles of 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min. A no-template control (NTC) was performed and *M. smegmatis* served as the positive control in every PCR run.

The sequences of the forward and reverse primers for the universal 16S assay were 5'-ACTCCTACGGGNGGCNGCA-3' and 5'-GTATTACCGCNNCTGC TGGCAC-3', respectively. The PCR conditions for this assay were identical to those for the mycobacterial 16S assay. Both these PCR assays were developed and optimized by Cheah (2010) and Haldar (unpublished), respectively.

### 3.10.3 Gel Analysis of PCR Amplicons

PCR amplicons from both the 16S assays were separated by electrophoresis on 2% w/v agarose gels. A volume of 10 µl for each amplicon was analyzed on the gel along with 1.5 µg of GeneRuler 100-bp DNA ladder. Gel electrophoresis was performed at 80 V for 30 min. After that, the gel was stained with ethidium bromide for 5-10 min and the PCR amplicons visualized under the UV transilluminator.

### 3.10.4 Gel Purification of PCR Amplicons

DNA bands on the gel of the correct size (173 bp for mycobacterial isolates and 199 bp for contaminant isolates) were excised with a scalpel. These amplicons were then purified using the Invisorb Spin DNA Extraction Kit according to the manufacturer's protocol. The purity ($A_{260}/A_{280}$) and concentration of the resulting DNA suspensions were determined using a nanophotometer; the former should be between 1.8 and 2.0, while the latter should be more than 10 ng/µl.

### 3.10.5 DNA Sequencing and Analyses

Purified PCR amplicons were sent to Medigene Sdn. Bhd. (Selangor, Malaysia) for DNA sequencing. The resulting DNA sequences were analyzed using the nucleotide-nucleotide Basic Local Alignment Search Tool (BLASTn) at the National Center for Biotechnology Information (NCBI) website. The BLASTn results for each isolate that give the lowest expect value (E-value) and highest percentage of identify were used for the final identification of the isolate, along with results from phenotypic analyses.

## 3.11 Long-term Storage of Isolates

Two loopfuls of pure colonies for each isolate were suspended in 0.5 ml of Middlebrook 7H9 broth and then mixed with 0.5 ml of 65% v/v glycerol solution. All isolate stocks were stored at -80 ºC.

# CHAPTER 4

# RESULTS

## 4.1 Properties of Samples

Upon visual inspection, the soils from the cow farm and the buffalo-feeding ground near KTAR were dark brown to black in color while those from the buffalo-feeding ground in Eastlake were reddish in appearance (Figure 4.1). Texture wise, the latter were relatively sandier than the former. The soil samples from the cow farm, Eastlake feeding ground, and KTAR feeding ground were tested to be neutral, slightly acidic, and acidic, respectively (Table 4.1). All the water samples were clear in appearance, with very minimal sediments observed in them. No pH measurements were made for them.

**Table 4.1: pH of soil samples from different locations and sites**

| Location | Site | pH |
|---|---|---|
| Cow farm | 1 | 7.31 ± 0.30 |
| | 2 | 7.25 ± 0.25 |
| | 3 | 6.79 ± 0.29 |
| Eastlake buffalo-feeding ground | 1 | 6.59 ± 0.06 |
| | 2 | 6.99 ± 0.26 |
| | 3 | 6.71 ± 0.26 |
| KTAR buffalo-feeding ground | 1 | 5.01 ± 0.58 |
| | 2 | 4.79 ± 0.04 |
| | 3 | 4.77 ± 0.01 |

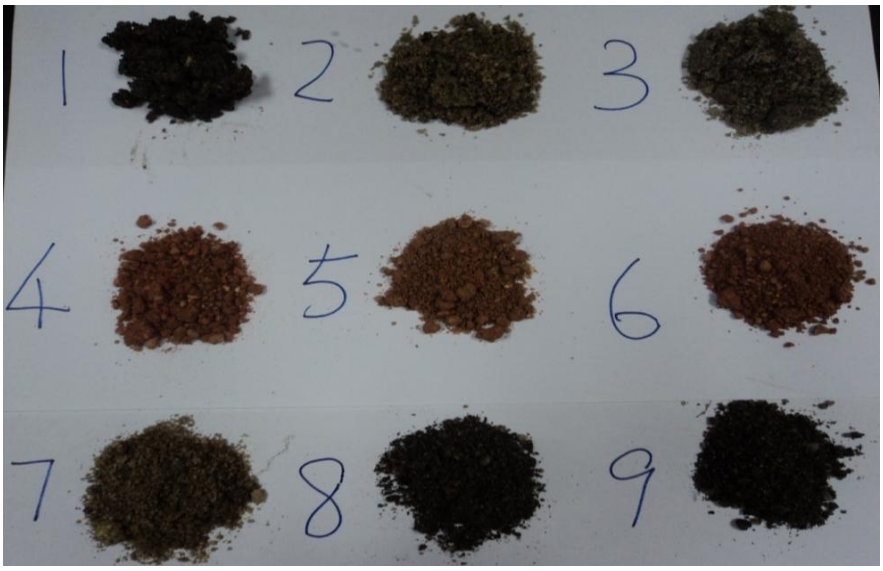


**Figure 4.1: Physical appearance of soil samples from different locations and sites**
Samples 1, 2, and 3 were collected from sites 1, 2, and 3, respectively, around the cow farm; samples 4, 5, and 6 were collected from sites 1, 2, and 3, respectively, around Eastlake feeding ground; samples 7, 8, and 9 were collected from sites 1, 2, and 3, respectively, around KTAR feeding ground.

## 4.2 Chemical Decontamination of Samples

### 4.2.1 Primary Decontamination

Samples from six out of 18 sampling sites yielded AFB-positive cultures; these were from three sampling locations (Table 4.3). All three soil samples from the cow farm were positive for AFB. The other three positive ones were water samples, in which one was from the lake near UTAR Block C and two were from the lake near UTAR Sport Complex. However, all six cultures were not of pure AFB isolates and therefore they were subjected to further decontamination in order to obtain pure AFB cultures.

**Table 4.2: Isolation of AFB in cultures following primary decontamination**

| Location | Sample type | Site | Detection of AFB-positive culture† | | Isolate |
|---|---|---|---|---|---|
| | | | 30ºC | 37ºC | |
| Cow farm | Soil | 1 | + | - | A |
| | | 2 | - | + | B |
| | | 3 | - | + | D |
| Eastlake buffalo-feeding ground | Soil | 1 | - | - | |
| | | 2 | - | - | |
| | | 3 | - | - | |
| KTAR buffalo-feeding ground | Soil | 1 | - | - | |
| | | 2 | - | - | |
| | | 3 | - | - | |
| UTAR Block F river | Water | 1 | - | - | |
| | | 2 | - | - | |
| | | 3 | - | - | |
| UTAR Block C lake | Water | 1 | - | - | |
| | | 2 | + | - | C |
| | | 3 | - | - | |
| UTAR Sport Complex lake | Water | 1 | + | - | E |
| | | 2 | - | + | F |
| | | 3 | - | - | |

† +, detection of AFB-positive culture; -, no detection of AFB-positive culture

### 4.2.2 Secondary Decontamination

#### 4.2.2.1 Decontamination with Cetrimide

Different concentrations of cetrimide and durations of treatment were evaluated for decontamination of mixed primary cultures. Only isolates A and B yielded pure AFB cultures following 5-min treatment with 1% w/v cetrimide and 15-min treatment with 2% w/v cetrimide, respectively (Table 4.3).

**Table 4.3: Decontamination of mixed primary cultures with cetrimide**

| Concentration of cetrimide (%, w/v) | Duration of treatment (min) | Isolate† | | | | | |
|---|---|---|---|---|---|---|---|
| | | A | B | C | D | E | F |
| 1 | 5 | + | - | - | - | - | - |
| 1 | 15 | + | - | - | - | - | - |
| 2 | 5 | + | - | - | - | - | - |
| 2 | 15 | + | + | - | - | - | - |
| 2 | 30 | + | + | - | - | - | - |

† +, pure AFB culture; -, mixed culture

#### 4.2.2.2 Decontamination with NaOH

The four mixed cultures that failed cetrimide decontamination were subjected to NaOH decontamination; different concentrations of NaOH and durations of treatment were also evaluated. Only isolate C yielded pure AFB culture following 15-min treatment with 4% w/v NaOH (Table 4.4).

**Table 4.4: Decontamination of mixed primary cultures with NaOH**

| Concentration of NaOH (%, w/v) | Duration of treatment (min) | Isolate[†] | | | |
|---|---|---|---|---|---|
| | | C | D | E | F |
| 2 | 15 | - | - | - | - |
| 2 | 30 | - | - | - | - |
| 4 | 15 | + | - | - | - |
| 4 | 30 | + | - | - | - |

[†] +, pure AFB culture; -, mixed culture

#### 4.2.2.3 Decontamination with Antibiotic

The three mixed cultures that failed both cetrimide and NaOH decontamination were inoculated onto LJ slants containing 50 U/ml penicillin. Only Isolate D yielded pure AFB culture. Isolates E and F were lost in the end due to overgrowth of non-acid-fast organisms and were not tested further.

### 4.3 Phenotypic Identification of AFB Isolates

#### 4.3.1 Acid-fastness

Acid-fastness refers to the ability of mycobacteria to resist decolorization by acid-alcohol during ZN staining. This phenotypic feature was used to verify recovery of AFB during primary isolation. AFB retained the red color of carbol fuchsin while non-acid-fast organisms were stained blue by the counterstain methylene blue (Figure 4.2). Both red- and blue-stained cells were observed for

mixed cultures (Figure 4.2b). With regard to cell morphology, Isolates A, C, and D consisted of long rods, while Isolate B consisted of coccoid to long rods (Figure 4.3). ZN staining was also performed to determine the effectiveness of secondary decontamination of mixed primary cultures. Isolates E and F were heavily stained in blue, indicating large populations of non-acid-fast organisms, and were not tested further (Figure 4.4).

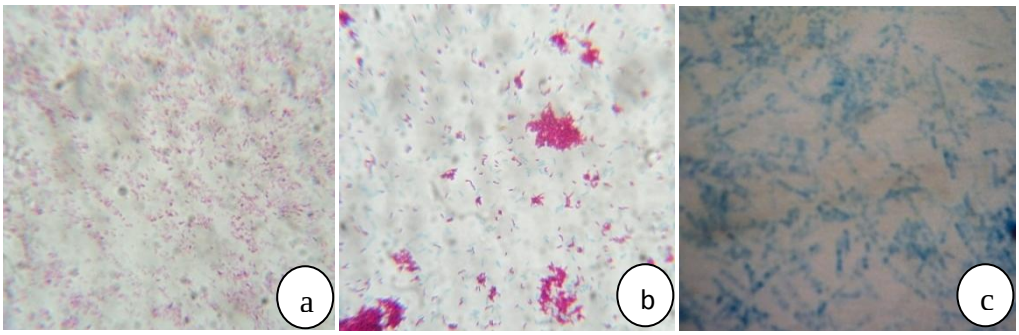


**Figure 4.2: ZN-stained isolates observed under oil immersion (1000×). a) Pure AFB isolates; b) mixed isolates; c) AFB-negative isolates.**

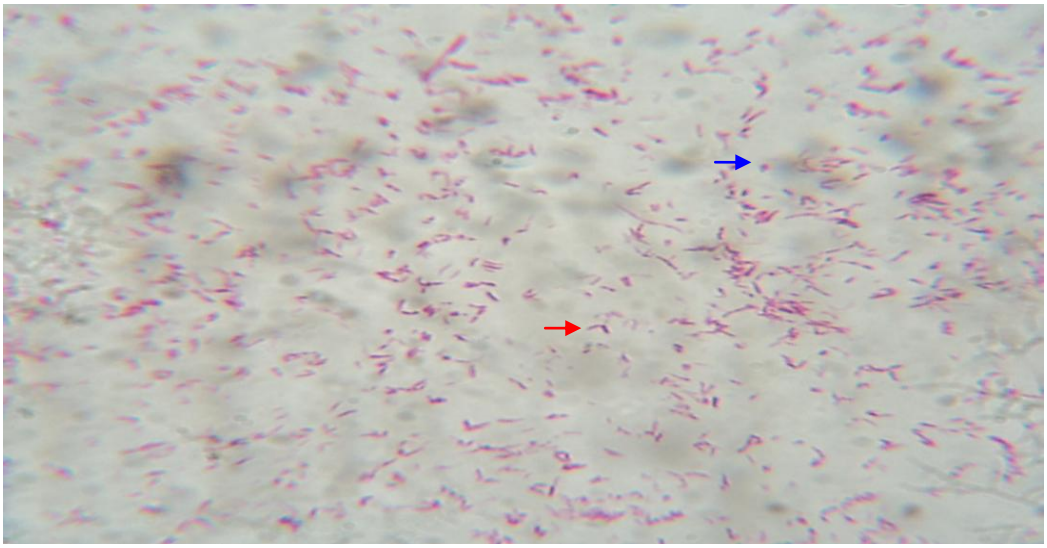

**Figure 4.3: Pleomorphic cell morphology of Isolate B observed under oil immersion (1000×)**
Coccoid and long rods are shown by blue and red arrows, respectively, for clarity.

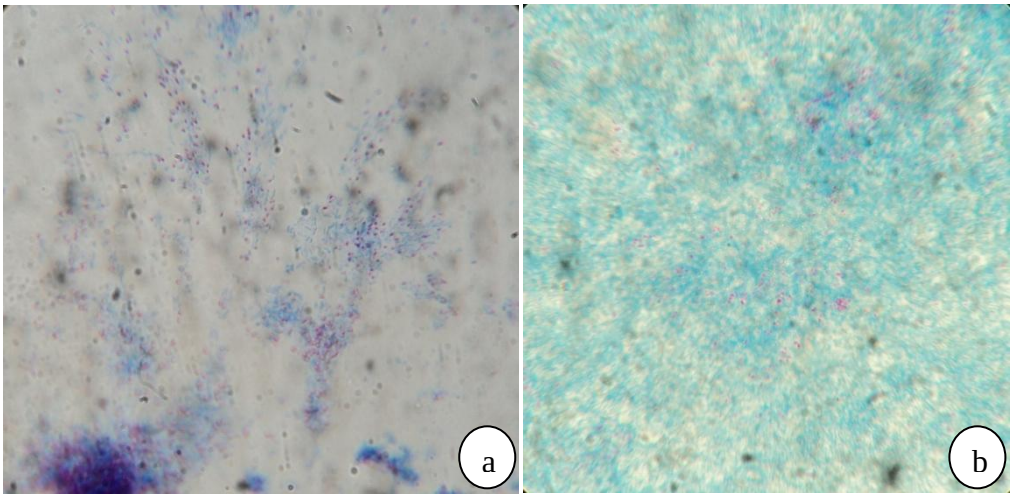


**Figure 4.4: ZN-stained Isolates E and F observed under oil immersion (1000×). a) Isolate E; b) Isolate F.**

### 4.3.2 Growth Rate

Growth rate of mycobacteria is the length of time taken to form colonies on solid media that are visible to the naked eye. Those that form visible colonies within seven days are called rapid growers while those that take more than seven days are called slow growers. The growth rate was determined during primary isolation. Isolates A and B formed visible colonies after five and seven days of incubation, respectively, and were therefore rapid growers. Isolates C and D were slow growers that formed visible colonies after 14 and 22 days, respectively.

### 4.3.3 Pigmentation and Colony Morphology

NTM can be classified according to their ability to form pigmented colonies in the presence and/or absence of light. Isolates A and D were nonphotochromogens; their colonies were nonpigmented and pale yellow in color (Figure 4.5a). Isolates B and C gave orange colonies under both bright and dark conditions, and were scotochromogens (Figure 4.5b). The test

reference *M. smegmatis* is a scotochromogen that gave orange-pigmented colonies. The texture of mycobacteria colony can be smooth or rough. Isolates A and D formed smooth colonies while Isolates B and C formed rough colonies. Other morphological features were not studied due to the inherent difficulty in discerning colonies from debris on the LJ slants (Pfyffer, Brown-Elliott & Wallace Jr., 2003).

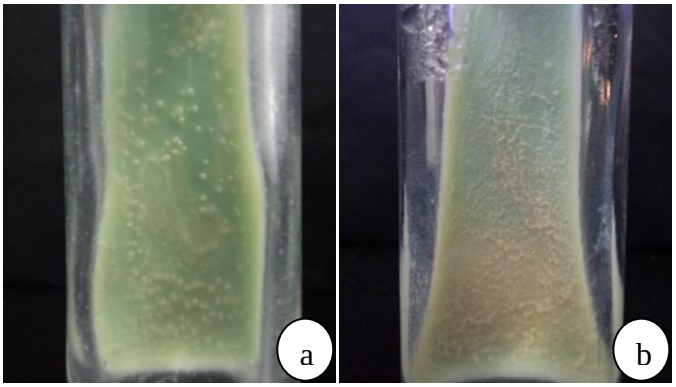


**Figure 4.5: Pigmentation and colony morphology of AFB isolates. a) Non-pigmented pale yellow, smooth colonies of Isolate A; b) orange-pigmented, rough colonies of Isolate B.**

### 4.3.4 Biochemical Features

Five biochemical tests were carried out: semiquantitative and thermostable catalase tests, urease test, salt tolerance test, and Tween 80 hydrolysis test. The overall results are summarized in Table 4.5. The results for the Tween 80 hydrolysis test were not reported as the negative control gave false positive result.

**Table 4.5: Biochemical profiles of the AFB isolates**

| Biochemical test | Isolate[†] | | | |
|---|---|---|---|---|
| | A | B | C | D |
| Semiquantitative catalase test (mm) | >45 | >45 | >45 | >45 |
| Thermostable catalase test | + | + | + | - |
| Urease test | + | + | - | + |
| Salt tolerance test | + | + | + | + |

[†] +, positive reaction; -, negative reaction

#### 4.3.4.1 Semiquantitative Catalase Test

This test was performed to assess for the presence of catalase activity and to semiquantitatively measure the amount of the enzyme produced. The latter was achieved by measuring the maximum height of the bubble column formed in the test tube; the threshold is set at 45 mm, above which isolates would be categorized as high producers and below which isolates would be grouped as low producers (Vincent, Brown-Elliott, Jost Jr & Wallace Jr, 2003). All the AFB isolates were high catalase producers (Table 4.5; Figure 4.6).

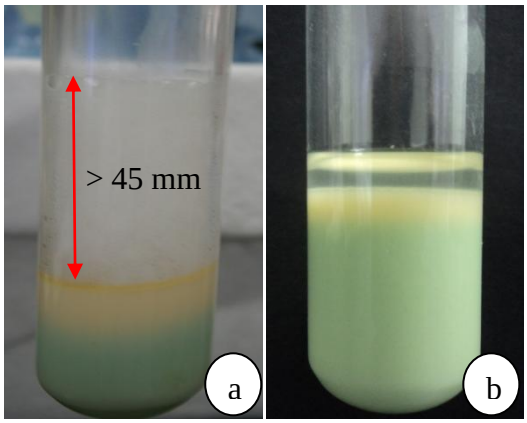


**Figure 4.6: Screening for semiquantitative catalase activity. a) Positive reaction of Isolate A showing bubble column of more than 45 mm in height; b) negative control.**
The height of bubble column is shown by red double-headed arrow for clarity.

#### 4.3.4.2 Thermostable Catalase Test

This test was performed to assess for the stability of catalase at 68 °C. Production of bubbles was observed for Isolates A, B, and C, which indicates the presence of heat-stable catalase (Table 4.5; Figure 4.7). Isolate D was negative for this test.

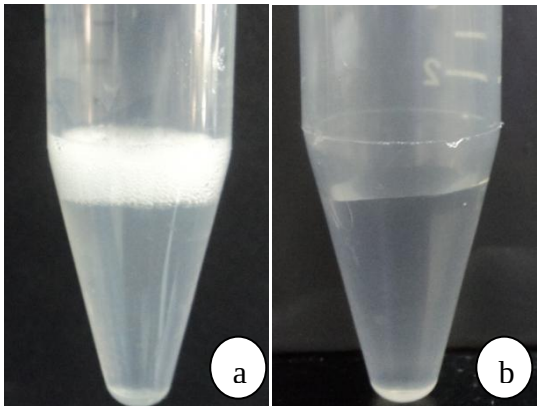


**Figure 4.7: Screening for thermostable catalase activity. a) Positive reaction of Isolate A showing bubble formation; b) negative reaction of Isolate D with no bubble formation.**

#### 4.3.4.3 Urease Test

This test was performed to assess for the presence of urease activity; a color change of the test suspension from yellow to pink or red would indicate a positive reaction. All the AFB isolates showed the presence of urease, except for Isolate C (Table 4.5; Figure 4.8). The low color intensity of the positive reaction was due to slight error in the procedure; the prepared urea broth was mistakenly diluted 10 times prior to use. The negative reaction should appear yellow in color.

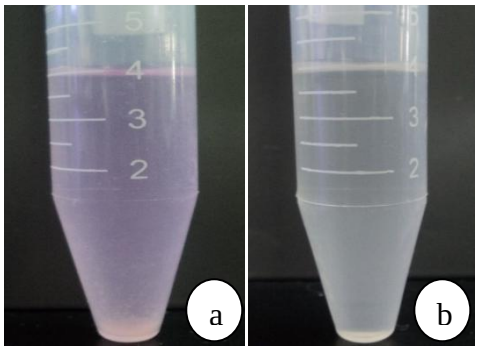


**Figure 4.8: Screening for urease activity. a) Positive reaction of Isolate A; b) negative reaction of Isolate C.**

#### 4.3.4.4 Salt Tolerance Test

This test was performed to assess for the tolerance of the AFB isolates to 5% w/v NaCl; growth of more than 50 colonies on the LJ slant supplemented with NaCl would indicate a positive result. All the isolates showed salt tolerance (Table 4.5; Figure 4.9).

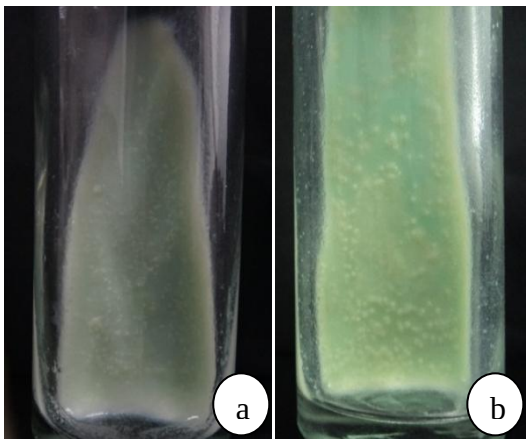


**Figure 4.9: Screening for salt tolerant characteristic. a) Positive result of Isolate D showing more than 50 colonies formed; b) growth of control on LJ slant without salt.**

#### 4.3.4.5 Tween 80 Hydrolysis Test

This test was performed to assess for the presence of Tween 80-hydrolyzing activity of lipase; a color change of the test suspension from orange to red would indicate a positive reaction. The "positive" results for all the AFB isolates were ambiguous, as the negative control gave a false positive reaction (Figure 4.10).

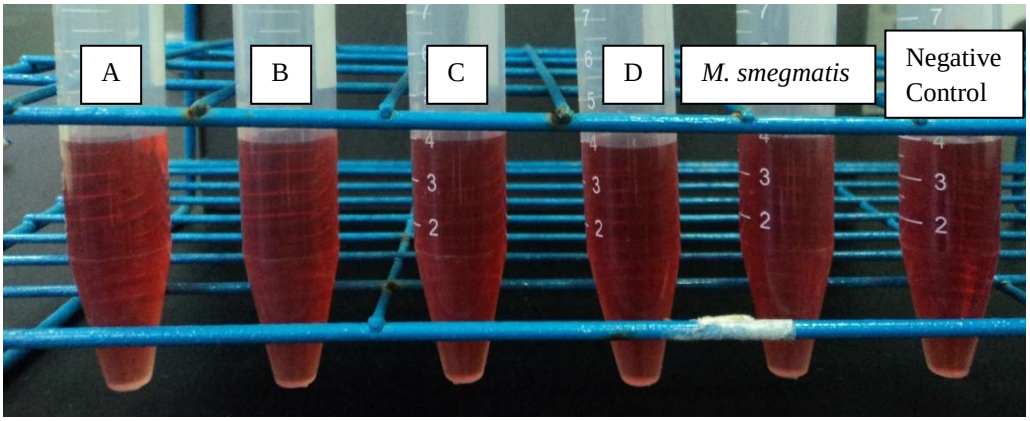


**Figure 4.10: Screening for lipase activity in the Tween 80 hydrolysis test.**

### 4.3.5 Mycobacteriophage Infection

D29 used for this test is a lytic mycobacteriophage that can infect a wide variety of fast- and slow-growing mycobacterial species; the end-point being formation of clear plaques (Froman, Will, & Bogen, 1954). Although the plates for Isolates A and D showed signs of phage infection, the plaques formed were turbid (Figure 4.11). Isolate B showed no evidence of phage D29 infection. Isolate C was not tested due to the inherent difficulty in obtaining homogenous culture suspension for lawn formation.

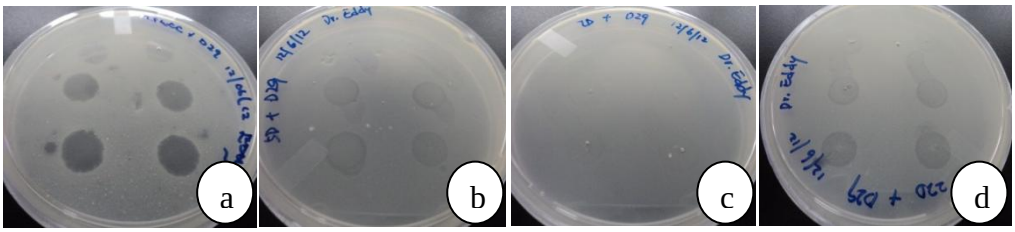


**Figure 4.11: Plaque appearance on lawns of mycobacterial cells. a) *M. smegmatis* (positive control); b) Isolate A; c) Isolate B; d) Isolate D.**

## 4.4 Genotypic Identification of AFB Isolates

### 4.4.1 Mycobacterial 16S rDNA PCR and Gel Analysis

Boiled DNA lysates from the AFB isolates were used as templates for the mycobacterial 16S PCR assay. The resulting amplicons were analyzed by electrophoresis on 2% w/v agarose gels. All the isolates yielded amplicons of the expected size of 173 bp (Figure 4.12). However, NTCs also gave gel bands of similar size, although the intensity of their brightness was very much lower than that for the AFB amplicons. DNA sequencing of NTC amplicons indicated that they were those of 16S rDNA from nonmycobacterial actinobacteria. This observation has been reported previously (Cheah, 2010). Test run of this PCR assay using a separate set of PCR reagents by a commercial laboratory resulted in similar outcome (data not shown).

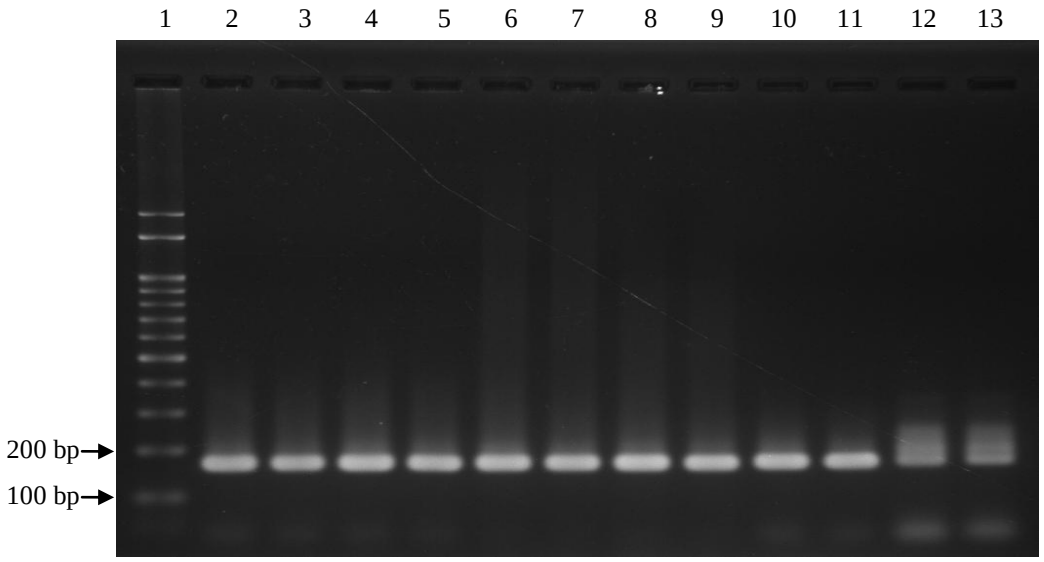


**Figure 4.12: Gel analysis of mycobacterial 16S amplicons**
Lane 1, GeneRuler 100-bp DNA ladder; lanes 2 and 3, Isolate A; lanes 4 and 5, Isolate B; lanes 6 and 7, Isolate C; lanes 8 and 9, Isolate D; lanes 10 and 11, *M. smegmatis* (positive control); lanes 12 and 13, NTC.

### 4.4.2 DNA Sequence Analysis of Mycobacterial 16S Amplicons

All purified amplicons showed $A_{260}/A_{280}$ values of 1.8-2.0 and their concentrations were more than 10 ng/µl. BLASTn analyses revealed that the resulting DNA sequences of Isolates A, B, and D corresponded to those of the mycobacterial 16S rRNA gene (Table 4.6). Interestingly, the DNA sequence for Isolate C matched the partial 16S rDNA sequence for *Norcadia* sp. The BLASTn results with the lowest E-value and highest percentage of identity for each isolate were selected and used for final species identification (Section 4.5). An example of good alignment between query and test mycobacterial DNA sequences is shown in Figure 4.13.

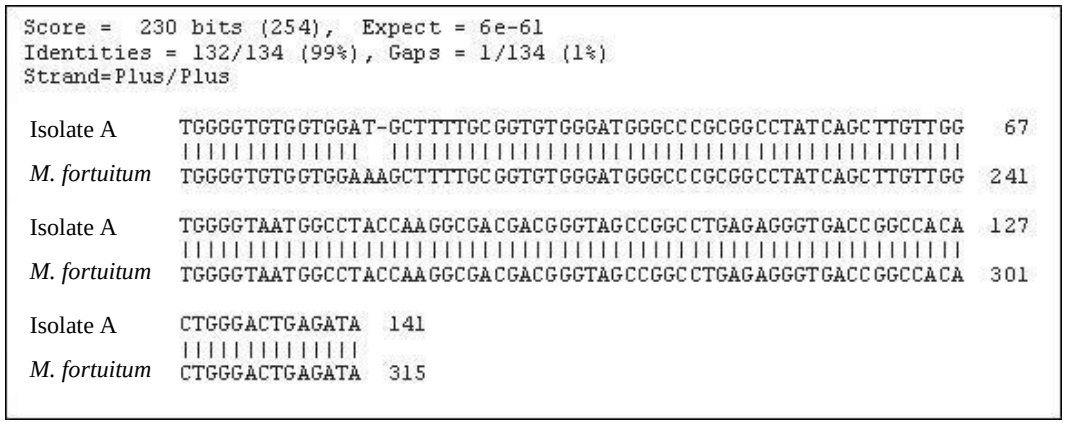


**Figure 4.13: Alignment of the partial 16S rDNA sequence of Isolate A with that of *M. fortuitum***

**Table 4.6: BLASTn analysis results for the AFB isolates**

| Isolate | BLASTn identity | Accession | Score (bits) | E-value | Identity (%) |
|---|---|---|---|---|---|
| A | 16S ribosomal RNA (*Mycobacteium llatzerense*) | NR_042280.1 | 230 | $6e^{-61}$ | 99 |
| | 16S ribosomal RNA (*Mycobacterium canariasense*) | NR_025737.1 | 230 | $6e^{-61}$ | 99 |
| | 16S ribosomal RNA (*Mycobacteium porcinum*) | NR_042920.1 | 230 | $6e^{-61}$ | 99 |
| | 16S ribosomal RNA (*Mycobacterium fortuitum*) | NR_042914.1 | 230 | $6e^{-61}$ | 99 |
| | 16S ribosomal RNA (*Mycobacteium aubagnense*) | NR_043238.1 | 230 | $6e^{-61}$ | 99 |
| | 16S ribosomal RNA (*Mycobacterium boenickei*) | NR_029036.1 | 230 | $6e^{-61}$ | 99 |
| B | 16S ribosomal RNA (*Mycobacterium setense*) | NR_044110.1 | 230 | $6e^{-61}$ | 99 |
| | 16S ribosomal RNA (*Mycobacteium senegalense*) | NR_042921.1 | 230 | $6e^{-61}$ | 99 |
| | 16S ribosomal RNA (*Mycobacterium mucogenicum*) | NR_042919.1 | 230 | $6e^{-61}$ | 99 |
| | 16S ribosomal RNA (*Mycobacteium septicum*) | NR_042916.1 | 230 | $6e^{-61}$ | 99 |
| | 16S ribosomal RNA (*Mycobacterium peregrinum*) | NR_042915.1 | 230 | $6e^{-61}$ | 99 |
| | 16S ribosomal RNA (*Mycobacteium fortuitum*) | NR_042913.1 | 230 | $6e^{-61}$ | 99 |
| | 16S ribosomal RNA (*Mycobacterium fallax*) | NR_041904.1 | 230 | $6e^{-61}$ | 99 |
| | 16S ribosomal RNA (*Mycobacteium conceptionense*) | NR_043239.1 | 230 | $6e^{-61}$ | 99 |
| | 16S ribosomal RNA (*Mycobacterium phocaicum*) | NR_043237.1 | 230 | $6e^{-61}$ | 99 |
| | 16S ribosomal RNA (*Mycobacteium frederiksbergense*) | NR_025393.1 | 230 | $6e^{-61}$ | 99 |
| | 16S ribosomal RNA (*Mycobacterium alvei*) | NR_024859.1 | 230 | $6e^{-61}$ | 99 |

**Table 4.6 (Continue…)**

| Isolate | BLASTn identity | Accession | Score (bits) | E-value | Identity (%) |
|---|---|---|---|---|---|
| C | 16S ribosomal RNA (*Nocardia sienata*) | NR_024825.1 | 203 | $1e^{-52}$ | 95 |
| | 16S ribosomal RNA (*Nocardia testacea*) | NR_041251.1 | 203 | $1e^{-52}$ | 95 |
| D | 16S ribosomal RNA (*Mycobacterium llatzerense*) | NR_042280.1 | 232 | $2e^{-61}$ | 99 |
| | 16S ribosomal RNA (*Mycobacterium canariasense*) | NR_025737.1 | 232 | $2e^{-61}$ | 99 |
| | 16S ribosomal RNA (*Mycobacterium porcinum*) | NR_042920.1 | 232 | $2e^{-61}$ | 99 |
| | 16S ribosomal RNA (*Mycobacterium fortuitum*) | NR_042914.1 | 232 | $2e^{-61}$ | 99 |
| | 16S ribosomal RNA (*Mycobacterium aubagnense*) | NR_043238.1 | 232 | $2e^{-61}$ | 99 |
| | 16S ribosomal RNA (*Mycobacterium boenickei*) | NR_029036.1 | 232 | $2e^{-61}$ | 99 |

### 4.5 Final Identification of AFB Isolates

Based on genotypic results alone, there would be multiple possible identities for most of these isolates (Table 4.6). In this case, consideration of both genotypic and phenotypic results in combination would narrow down the list of possible identities for each isolate. Phenotypic results for each of the isolates were compared to those for the species selected from the BLASTn analysis; the phenotypic characteristics that were not reported previously for the latter were excluded from analyses. The results of the final identification are shown in Table 4.7. The most possible identities of Isolates A, B, C, and D were *M. fortuitum*, *M. senegalense*, *Norcadia* sp., and *M. porcinum*, respectively.

### 4.6 Identification of Non-acid-fast Organisms in Primary Cultures

Non-acid-fast organisms observed following primary decontamination posed a challenge to the isolation and identification of mycobacteria. Therefore, further characterization of these "contaminants" is of great value to identify more stringent decontamination methods to eliminate them. Contaminants A, B, and E were Gram-negative rods while C and D were Gram-positive rods (Figure 4.14). Boiled DNA lysates from these contaminants were subjected to the universal 16S assay and all resulting amplicons showed bands of the expected size of 199 bp upon gel analysis (Figure 4.15). BLASTn analyses of the amplicon sequences for three isolates revealed similarity to the partial 16S rDNA sequences of two bacterial species, namely *Paenibacillus* sp. and *Bacillus pumilus* (Table 4.8). The other two isolates were annotated as soil bacterium 3V-02 in the NCBI database.

**Table 4.7: Final identification of the AFB isolates based on their phenotypic and genotypic results**

**a) Isolate A**

| Species | Phenotypic feature[†] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Growth rate[‡]** | **Growth temperature (ºC)** | **Pigment production[§]** | **Colony texture[*]** | **Cell morphology** | **Semi-quantitative catalase test (mm)** | **Thermostable catalase test** | **Urease test** | **Salt tolerance test** | **Similarity (%)** |
| *M. llatzerense* | F | 22-30 | N | NR | NR | <45 | - | - | - | 42.9 |
| *M. canariasense* | F | 30-37 | N | Sm | NR | NR | + | NR | - | 83.3 |
| *M. porcinum* | F | 24-42 | N | R/Sm | Long rods | >45 | + | - | + | 88.9 |
| *M. fortuitum* | F | 28-30 | N | R/Sm | Beaded to long rods | >45 | + | + | + | 100.0 |
| *M. aubagnense* | F | 24-37 | N | NR | NR | NR | - | - | - | 50.0 |
| *M. boenickei* | F | 35 | N | NR | Coccoird to long rods | >45 | + | + | + | 77.8 |
| Isolate A | F | 30 | N | Sm | Long rods | >45 | + | + | + | |

The most possible species is *M. fortuitum*.

[†] NR, not reported; +, positive reaction; -, negative reaction
[‡] F, fast growth; S, slow growth
[§] N, nonpigmented; P, pigmented
[*] R, rough; Sm, smooth

**b) Isolate B**

| Species | Phenotypic feature[†] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Growth rate[‡]** | **Growth temperature (ºC)** | **Pigment production[§]** | **Colony texture[*]** | **Cell morphology** | **Semi-quantitative catalase (mm)** | **Thermostable catalase** | **Urease test** | **Salt tolerance** | **Similarity (%)** |
| *M. setense* | F | 25-37 | N | Sm | Coccoid to long rods | NR | + | + | + | 75.0 |
| *M. senegalense* | F | 25-37 | N | R | Coccoid to long rods | NR | NR | + | + | 85.7 |
| *M. mucogenicum* | F | 24-37 | N | Sm | Coccoid to long rods | >45 | - | - | - | 44.4 |
| *M. septicum* | F | 28-35 | N | Sm | Coccoid to long rods | NR | NR | + | + | 57.4 |
| *M. peregrinum* | F | 28-37 | N | Sm | Coccoid to long rods | >45 | + | + | + | 77.8 |
| *M. fortuitum* | F | 28-30 | N | R/Sm | Beaded to long rods | >45 | + | + | + | 66.7 |
| *M. fallax* | F | 30-37 | N | R | Coccoid rods | <45 | - | - | - | 44.4 |
| *M. conceptionense* | F | 25-37 | N | NR | NR | NR | NR | - | + | 60.0 |
| *M. phocaicum* | F | 24-37 | N | NR | NR | NR | - | - | - | 33.3 |
| *M. frederiksbergense* | F | 15-37 | P | Sm | Coccoid rods | >45 | NR | - | + | 75.0 |
| *M. alvei* | F | 25-37 | N | R | Coccoid rods | >45 | + | + | - | 77.8 |
| Isolate B | F | 37 | P | R | Coccoid to long rods | >45 | + | + | + | |

The most possible species is *M. senegalense*.

## c) Isolate D

| Species | Phenotypic feature[†] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Growth rate[‡]** | **Growth temperature (ºC)** | **Pigment production[§]** | **Colony texture[*]** | **Cell morphology** | **Semi-quantitative catalase (mm)** | **Thermostable catalase** | **Urease test** | **Salt tolerance** | **Similarity (%)** |
| *M. llatzerense* | F | 22-30 | N | NR | NR | <45 | - | - | - | 28.6 |
| *M. canariasense* | F | 30-37 | N | Sm | NR | NR | + | NR | - | 50.0 |
| *M. porcinum* | F | 24-42 | N | R/Sm | Long rods | >45 | + | - | + | 77.7 |
| *M. fortuitum* | F | 28-30 | N | R/Sm | Beaded to long rods | >45 | + | + | + | 66.7 |
| *M. aubagnense* | F | 24-37 | N | NR | NR | NR | - | - | - | 50.0 |
| *M. boenickei* | F | 35 | N | NR | Coccoird to long rods | >45 | + | + | + | 62.5 |
| Isolate D | S | 37 | N | Sm | Long rods | >45 | - | + | + | |

The most possible species is *M. porcinum*.

The physical appearances of inoculated LJ slants could sometimes indicate contamination by non-acid-fast organisms. These include liquefaction and deterioration of medium, increase in the color intensity of malachite green, and release of foul smell. ZN staining showed affected slants were heavily contaminated. The different levels of slant liquefaction and deterioration are shown in Figure 4.16.

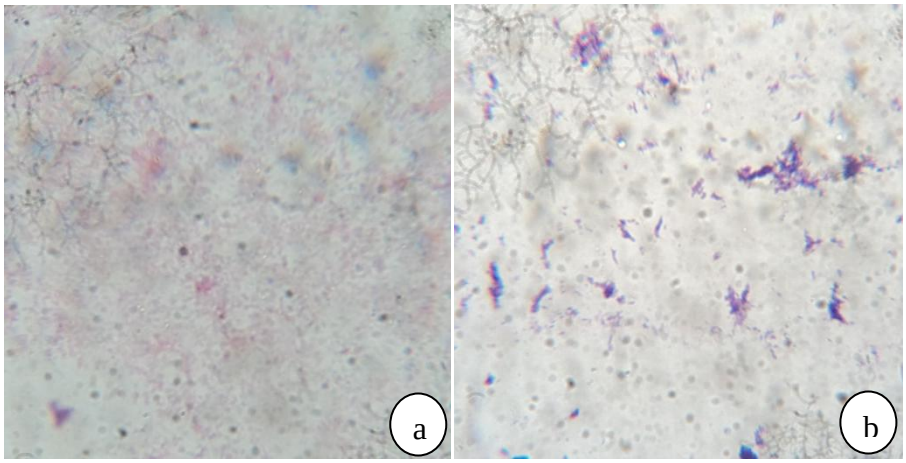


**Figure 4.14: Gram-stained contaminant organisms observed under oil immersion (1000x). a) Gram-negative rods of Organism A; b) Gram-positive rods of Organism C.**

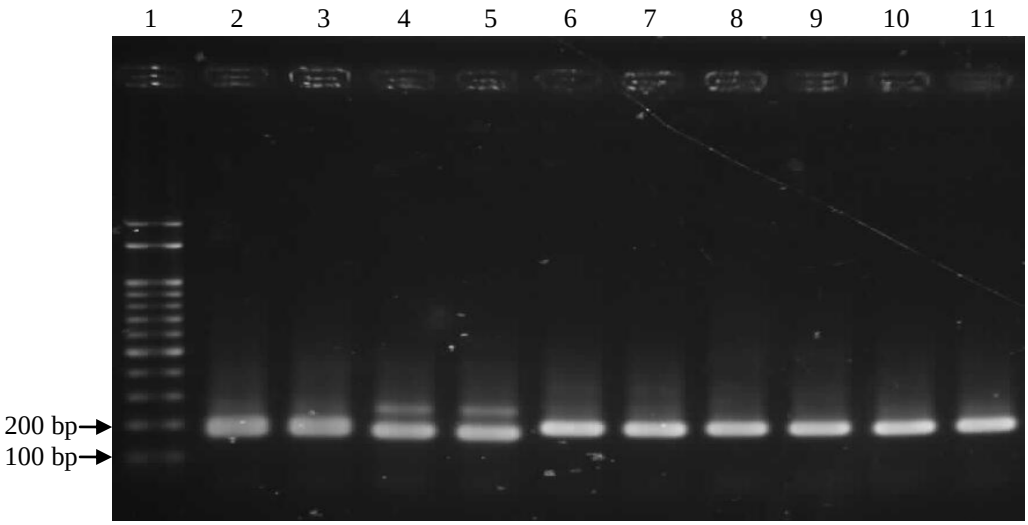


**Figure 4.15: Gel analysis of 16S amplicons of contaminant organisms from primary cultures**
Lane 1, GeneRuler 100-bp DNA ladder; lanes 2 and 3, Organism A; lanes 4 and 5, Organism B; lanes 6 and 7, Organism C; lanes 8 and 9, Organism D; lanes 10 and 11, Organism E.

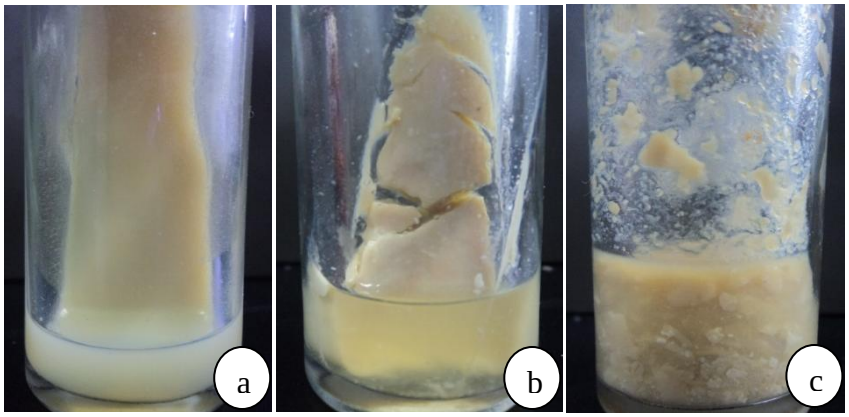


**Figure 4.16: Different levels of liquefaction and deterioration of contaminated LJ slants. a) Slight liquefaction; b) liquefaction and deterioration; c) severe liquefaction and deterioration causing LJ slant to collapse.**

**Table 4.8: BLASTn analysis results for contaminant organisms from primary cultures**

| Contaminant | BLAST identity | Accession | Score (bits) | E-value | Identity (%) |
|---|---|---|---|---|---|
| A | 16S ribosomal RNA (*Paenibacillus* sp.) | AY728023.1 | 257 | $1e^{-65}$ | 95 |
| B | 16S ribosomal RNA (Soil bacterium 3V-02) | EU839075.1 | 289 | $5e^{-75}$ | 99 |
| C | 16S ribosomal RNA (*Bacillus pumilus* strain BAB1328) | JQ389662.1 | 278 | $1e^{-71}$ | 97 |
| | 16S ribosomal RNA (*Bacillus pumilus* strain BAB329) | JQ389618.1 | 278 | $1e^{-71}$ | 97 |
| | 16S ribosomal RNA (*Bacillus pumilus* isolate 7BR11) | AY792029.1 | 278 | $1e^{-71}$ | 97 |
| D | 16S ribosomal RNA (*Bacillus pumilus* isolate 7BR11) | AY792029.1 | 206 | $4e^{-50}$ | 90 |
| E | 16S ribosomal RNA (Soil bacterium 3V-02) | EU839075.1 | 289 | $5e^{-75}$ | 99 |

# CHAPTER 5

# DISCUSSION

## 5.1 Sampling and Preliminary Assessment of Sample Properties

In this project, soils from bovid-contacted grounds and water from a river and lakes were sampled and analyzed for the presence of NTM species. All the soil samples were collected from the depth of approximately 3 cm below ground surface to minimize the detrimental effects of various environmental stresses. Even tough microorganisms like mycobacteria are not spared from killing by exposure to UV radiation, desiccation, and heat (Wilkins, 2008). Although UV light exposure is undesirable for surface water samples, the relatively higher heat capacity of water can minimize the effect of heat on mycobacteria. The pH of the soil samples ranged from 4.77 to 7.31; red soils were more acidic than dark ones as expected. Falkinham III (2002) reported that environmental mycobacteria grow over a wide pH range, and therefore pH might not have strong influence on the distribution of mycobacteria in the environment. One important factor to be considered is the nutrient levels of soils in which dark-colored ones generally have more nutrients than red ones.

## 5.2 Isolation of NTM from Soil and Water Samples

### 5.2.1 Primary Decontamination and Isolation

All samples were decontaminated with a combination of NaOH and the detergents SDS and cetrimide, as described by Parashar *et al.* (2004). These chemical agents function to lyse bacterial cells by physically disrupting their

cell membrane. Cetrimide can also kill bacterial cells by inhibiting the functions of their cell wall and cytoplasmic membrane (Ohta, Kondo, Kawada, Teranaka & Yoshino, 2008). Mycobacteria in the samples, if present, would survive this harsh chemical treatment due to the possession of a thick waxy cell envelope (Lanéelle & Daffé, 2009). However, different mycobacterial species differ in their ability to resist these chemical agents, depending on their cell wall composition (Falkinham III, 2002). Furthermore, variation in the resistance of mycobacterial cells in different physical and physiological states is also possible. With regard to the former, cells that are physically stressed or injured would be relatively more sensitive to the chemical treatment.

Only six out of 18 decontaminated samples yielded AFB-positive cultures (Table 4.2). These were from three out of six locations of study in which two were ex-tin-mining lakes and another was a cow farm. Successful isolation of AFB from all three soil samples from the cow farm and two water samples from the lake near to the UTAR Sport Complex suggests homogenous distribution of mycobacteria in these locations. Only one water sample from the lake near to UTAR Block C gave AFB-positive culture.

The presence of AFB in the soil samples from the buffalo-feeding ground near to KTAR and water samples from the river near to UTAR Block F could not be determined due to heavy growth of non-AFB organisms in the cultures; some of the LJ slants were liquefied and deteriorated. This might imply that the former contained very high levels of microorganisms and the decontamination method used was not stringent enough to control them. Nevertheless, the

presence of large microbial populations in acidic soils would be rather uncommon. The lack of microbial growth in the cultures for soil samples from the buffalo-feeding ground in Eastlake was very likely due to the decontamination procedure being too stringent for these samples. Additionally, the dry condition of these soils could be another reason for the negative isolation; moisture of soil is an important factor that influences the survival and growth of many bacterial species, including mycobacteria (Rahbar *et al.*, 2010).

### 5.2.2 Secondary Decontamination and Isolation

The AFB-positive cultures from primary isolation also contained non-AFB organisms, and therefore secondary decontamination was performed in order to obtain pure AFB cultures. Three different methods were used: cetrimide decontamination, NaOH decontamination, and inoculation onto penicillin-supplemented LJ media. Cetrimide decontamination was successful for Isolates A and B; the latter contaminants showed relatively higher resistance (Table 4.3). With NaOH decontamination, only Isolate C yielded pure AFB culture; the remaining three isolates still gave mixed cultures. The ineffectiveness of NaOH decontamination for heavily contaminated samples has been reported previously (McClean *et al.*, 2011). Only Isolate D yielded pure AFB culture on LJ slant supplemented with penicillin; Isolates E and F still gave mixed cultures and were not tested further. Wenzler, Schmidt-Eisenlohr, and Daschner (2003) reported that penicillin is only effective against Gram-positive bacteria; the inclusion of another antibiotic that is active against Gram-negative bacteria might be beneficial for heavily contaminated samples. All Isolates A, B, and D were from the same sampling location; it seems

plausible that different sites within the same area might harbor different levels of microorganisms.

There seems to be a trend in the power of decontamination for the three methods tested, with the lowest being cetrimide decontamination and the highest being the use of penicillin-supplemented medium. Perhaps a combination of chemical decontamination and subsequent inoculation onto penicillin-supplemented media might succeed in obtaining pure AFB cultures from Isolates E and F. Alternatively, more stringent methods can be evaluated, but the balance between power of decontamination and susceptibility of mycobacteria must be taken into consideration.

In general, this study assumed homogenous distribution of microorganisms in each sample, which might not be always true, especially for soil samples. In this case, multiple analyses for each sample would have minimized this limitation. Another limitation that should be taken into consideration is microbial loss during storage and processing.

### 5.3 Phenotypic Identification of AFB Isolates

#### 5.3.1 Acid-fastness

The presence of mycobacteria in test cultures was determined via ZN staining. Mycobacterial cells are said to be acid-fast because they retain the carbol fuchsin dye upon decolorization with acid-alcohol, and therefore appeared red in color under the microscope. In addition to the heat treatment during ZN staining, the relatively higher solubility of carbol fuchsin in cell wall lipids

facilitates movement of dye molecules into the cells (Mehrotra & Sumbali, 2009). Mycolic acids are thought to be the major lipids responsible for acid-fastness in mycobacteria (Gupte, 2006). Non-acid-fast organisms in the test cultures, if present, were stained blue by the counterstain methylene blue. The possibility of false negative staining results due to loss of acid-fastness among physically or chemically stressed mycobacterial cells warrants future investigation (Deb *et al.*, 2009).

Although Isolate C was identified as *Norcadia* sp., the positive ZN staining did not come as a surprise as partial acid-fastness has been reported for nonmycobacterial actinobacteria (Winn *et al.*, 2006). To distinguish between these actinobacterial species and mycobacteria, Gram staining would be useful. The former are true Gram positives whereas the latter would appear either beaded or almost as negative against the counterstained background during Gram staining, as the crystal violet in the dye does not readily penetrate their thick waxy cell envelope (Winn *et al.*, 2006).

The AFB in three positive cultures showed long and slender rods in which some appeared curved or bent. Coccoid form was also observed, in the case of Isolate B. According to Pfyffer *et al.* (2003), NTM may appear pleomorphic in which their cells can assume several different shapes.

### 5.3.2 Growth Rate

Among the four AFB isolates, two were rapid growers while the other two were slow growers. One important factor that influences the growth rate of

bacteria is the copy number of the 16S rRNA gene that is present. Bercovier, Kafri, and Sela (1986) reported that slow-growing mycobacterial species have only one copy of the 16S rRNA gene whereas rapid-growing ones, except for *M. chelonae* and *M. abscessus*, have two copies of this gene. The reported growth rate for the species identified for Isolate D is contradictory to that observed in this study; the former assigns this isolate to be that of a rapid-growing species. However, Lorian (2005) documented that some rapid growers may take much longer than seven days to be detected during primary isolation. Furthermore, the need for recovery following harsh chemical treatment could infer that the observed growth rates were overestimated in this study. The speed and ability of recovery could potentially be species-dependent, which might explain the ambiguous result for Isolate D.

### 5.3.3 Pigmentation and Colony Morphology

The Runyon Classification System groups NTM according to their ability to form pigmented colonies under different incubation conditions. Based on this system, two AFB isolates, A and D, were classified as nonchromogens while the other two are scotochromogens. The colonies of the former were nonpigmented and pale yellow in color, while the latter formed orange-pigmented colonies under both bright and dark conditions. The pigments of *Mycobacterium* spp. are carotenoid pigments that do not usually diffuse and vary from buff to yellow to orange in color (Shimeld & Rodgers, 1999). The test reference *M. smegmatis* is a late scotochromogen as it exhibited orange-pigmented colonies only after a week of growth, as reviewed by García-Agudo, Jesús, Rodrízguez-Iglesias, and García-Martos (2011).

Apart from color, the texture of AFB colonies was also observed and recorded during this study. According to Shimeld and Rodgers (1999), the colonies of mycobacteria can be either smooth or rough in texture. Isolates A and D formed smooth colonies while Isolates B and C formed rough colonies. The colonies for fast-growing species are usually smooth texture, and those for slow growers are usually rough. The existence of rough and smooth variants might explain the contradictory results for Isolates B and D, respectively (Leao *et al.*, 2004).

### 5.3.4 Biochemical Tests

#### 5.3.4.1 Catalase Tests

All the AFB isolates produced catalase, an enzyme that splits hydrogen peroxide into water and oxygen. *Mycobacterium* spp. are generally catalase-positive, apart from isoniazid-resistant strains of Mtb and *M. bovis*. However, the amount of the enzyme produced varies among different species, and this can be measured by the semiquantitative catalase test (Garcia & Isenberg, 2010). This test reflects the differences in the enzyme kinetics in which the amount of catalase produced is directly proportional to the height of the bubble column formed. All the test isolates were high catalase producers. According to Winn *et al.* (2006), some forms of catalase are inactivated by heating at 68 ºC for 20 min, and this is exploited for further characterization of mycobacterial species. All the AFB isolates produced heat-stable catalase, except for Isolate D.

#### 5.3.4.2 Urease Test

Only two of the AFB isolates, A and B, were positive for urease, an enzyme that hydrolyzes urea to ammonia and $CO_2$. In positive reactions, the production of ammonia increased pH of the media to above 8.2, and caused the color change of phenol red from yellow to pink or red. The delayed color development for test with Isolate D is considered a false positive reaction, since the negative control also changed color around the same time. These were very likely due to nonmicrobial degradation of urea in the broths. The urease test is useful in distinguishing between scotochromogens and nonchromogens; the former are generally urease-positive while the latter are urease-negative (Vincent *et al.*, 2003). Although Isolate C is a scotochromogen, the fact that it is a nocardia might explain the negative urease reaction (Seo & Lee, 2006).

#### 5.3.4.3 Salt Tolerance Test

All the AFB isolates yielded significant growth (more than 50 colonies) in LJ media containing 5% w/v NaCl, and were therefore salt tolerant. Conville and Witebsky (1998) reported that many species of rapidly growing mycobacteria are salt tolerant. This observation is consistent with the final identities of the three mycobacterial isolates. Isolate C, a slow-growing *Nocardia* sp., also showed salt tolerance.

#### 5.3.4.4 Tween 80 Hydrolysis Test

This test was not functioning during this study, judging from the false positive reaction for the negative control; the results were excluded from the final

identification. In principle, this test screens for the hydrolysis of Tween 80 to oleic acid and polyoxyethylated sorbitol by mycobacterial lipase. Phenol red in the test medium is bound by intact Tween 80 and has an orange color. If Tween 80 is hydrolyzed, phenol red is no longer bound and the test medium will develop a red color. The development of red color in pre-inoculated test media suggests that Tween 80 had been degraded. Repetition of the test with Tween 80 stock from a different laboratory resulted in similar outcome. This problem warrants further investigation.

### 5.4. Mycobacteriophage Infection

Two out of the three AFB isolates tested, A and D, were successfully infected by the lytic mycobacteriophage, D29. This is a mycobacterium-specific phage that infects both fast- and slow-growing species; the end-point being the formation of clear plaques (Froman *et al.*, 1954). However, the plaques that resulted from the infection of Isolates A and D were turbid in appearance, which are suggestive of lysogenic events. Indeed, Ford, Sarkis, Belanger, Hendrix, and Hatfull (1998) reported that lysogenization by D29 is possible in mycobacterial strains containing the plasmid-derived L5 repressor gene. The repressor protein expressed from this gene binds to an operator site overlapping the early lytic promoter, thereby maintaining stable lysogeny (Brown, Sarkis, Wadsworth & Hatfull, 1997). Alternatively, some mycobacterial cells in Isolates A and D could have already been lysogenized by lysogenic mycobacteriophages such as L5, and were therefore immune to subsequent infection by D29. The absence of plaques on the lawn of Isolate B indicates no infection by phage D29. The presence of phage-resistant or -nonresponsive

mycobacterial cells in this isolate could be a plausible explanation in this case. Besides that, the inability of D29 to form plaques in Isolate B could be an alternative explanation (Rybniker, Kramme & Small, 2006).

### 5.5 Genotypic Identification of AFB Isolates

All the AFB isolates yielded 16S amplicons of the expected size on gel analysis. Although Isolate C was later on identified as *Nocardia* sp., amplification of 16S rRNA genes of nonmycobacterial actinobacteria by the mycobacterial 16S primers has been reported previously (Cheah, 2010). The NTCs also gave gel bands of similar size to that of mycobacterial amplicons, although the intensity of their brightness was relatively lower. This observation has been reported by Cheah (2010), in which the investigation was inconclusive. The resulting NTC amplicons were sequenced to be those of the 16S rRNA gene of nonmycobacterial actinobacteria, consistent with the potential of these organisms to produce amplicons with the mycobacterial 16S primers. Since nonmycobacterial actinobacteria are commonly found in environmental reservoirs (Winn *et al*., 2006), it is possible that the water used in the PCR contained the DNAs of these organisms. However, the unambiguous sequencing results for the PCR positive controls should rule out any undesirable effects of this problem on the genotypic identification of mycobacterial isolates. Furthermore, preferential amplification of mycobacterial DNA by the 16S primers and interruption of nonspecific priming in mixed samples has been reported (Cheah, 2010).

Upon BLASTn analyses, the DNA sequences of the AFB amplicons showed high similarity to those of the mycobacterial 16S rRNA gene found in the NCBI database. However, analyses for most isolates yielded several possible identities of the same degree of homology and identity each (Table 4.6).

### 5.6 Final Identification of AFB Isolates

Both the genotypic and phenotypic identification results were interpreted simultaneously to facilitate final assignment of species identities for the AFB isolates. In this context, the BLASTn species with phenotypic characteristics that matched best (determined based on percentage of similarity) to those tested for the query AFB isolate would be flagged as its final identity (Table 4.7). Using this identification strategy, the possible identities for Isolates A, B, and D were determined to be *M. fortuitum*, *M. senegalense*, and *M. porcinum*, respectively. Isolate C was identified to be *Nocardia* sp.; the species identity was not determined as the set of biochemical tests performed was tailored for mycobacterial identification. The isolation of a *Nocardia* sp. in this study does not come as a surprise, as frequent isolation of this organism during culture of mycobacteria has been documented (Saubolle and Sussland, 2003).

Careful interpretation of phenotypic results for final species identification is of great importance and should take into account the possibility of strain variation and convergent profiles among different species (Vincent *et al.*, 2003). Horizontal gene transfer among different mycobacterial strains and species could account for these events (Nyuyen, Piastro, Gray & Derbyshire, 2010; Rosas-Magallanes *et al.*, 2006).

### 5.7 Assessment of Health Impacts of NTM Isolates on Human and Animal Hosts

NTM are distinguished from the members of the Mtb complex and *M. leprae* by the fact that they are not obligate pathogens but are true inhabitants of the environment (Primm *et al*., 2004). However, NTM have been reported to cause localized or disseminated disease, depending on local predisposition or degree of immune deficit in infected hosts (Katoch, 2004). According to Primm *et al.* (2004), the major mode of NTM transmission to human hosts is via environmental aerosols.

*M. fortuitum* has been reported to cause infections in patients undergoing cardiac bypass surgery and mammaplasty, and disseminated infections in immunocompromised patients (Bercovier and Vincent, 2001). In the case of the former, patients are subjected to increased risk of localized skin or soft tissue infections (Primm *et al.*, 2004). *M. fortuitum* infection has also been reported in wild animals; these include wild boars, swamp buffaloes, seals, armadillos, reptiles, amphibians, and several invertebrates (Bercovier & Vincent, 2001). *M. senegalense* is recognized as the principal pathogen of bovine farcy, which is a chronic skin and lymphatic disease affecting cattle in East and Central Africa (Oh *et al.,* 2005). In human host, catheter-related bloodstream infection by this NTM species was reported in a cancer patient in Korea (Oh *et al.* 2005). *M. porcinum* can cause chronic lung infection in human and lymphadenitis in swine (Wallace Jr. *et al.*, 2004).

### 5.8 Identification of Non-acid-fast Organisms in Primary Cultures

The presence of rapidly growing, non-acid-fast organisms in primary cultures posed a problem to the detection and isolation of slower growing, mycobacterial species. Preliminary information on these "contaminants" would facilitate development and evaluation of suitable decontamination methods to eliminate them. A few of these contaminants were identified during this study; most of them were Gram-positive rods from the genera *Bacillus* and *Paenibacillus*. It is highly possible that these bacilli survived the decontamination process via the formation of endospores. Alternatively, these contaminants might be airborne and could have contaminated the cultures post-decontamination. Indeed, the isolation of Gram-positive bacilli from the air has been reported (Rivas, Mateos, Martínez-Molina, and Velázquez, 2005). Inclusion of negative controls in future processing could assist in identifying possible sources of these contaminants.

The physical appearance of inoculated LJ slants could be indicative of contamination of cultures. One major observation in this study was liquefaction and deterioration of contaminated slants, which could be due to the proteolytic activity of certain contaminant species. Another observation was the increase in the green color intensity of contaminated slants, which was due to dissociation of malachite green from the egg when the medium pH was lowered by acidic byproducts of metabolism. Pure mycobacterial cultures also showed this observation at early incubation stage, but the color-intensified media were gradually decolorized over prolonged incubation period because

mycobacteria reduced the malachite green and sequestered the dye molecules in the lipid fraction of their cells (Jones & Falkinham III, 2003).

## 5.9 Future Works

### 5.9.1 Sample Analysis

The physical, chemical, and microbial properties of environmental samples could have strong influence on the efficacy of decontamination methods used. A thorough analysis of these properties would then provide information on the type and stringency of decontamination method suitable for the sample of interest.

### 5.9.2 Control of Contamination

#### 5.9.2.1 Germination of Endospores Prior to Decontamination Treatment

Most contaminants observed in this study were spore-forming, Gram-positive rods. The highly resistant endospores can survive harsh decontamination treatment, and therefore prior germination in nutrient-rich medium like LB or nutrient broth should be considered in future studies to increase their susceptibility to decontamination treatment.

#### 5.9.2.2 Use of Antibiotic-supplemented LJ Media in Routine Cultures

Routine use of these “super selective” media for culture would minimize the incidence of contamination. In fact, LJ media supplemented with penicillin and nalidixic acid have been used to isolate Mtb from clinical specimens (Gruft and Loder, 1971). However, a study to evaluate the effect of these media on the detection and isolation of mycobacteria in environmental samples is necessary.

### 5.9.3 Biochemical Tests

The set of biochemical tests conducted should be expanded to improve the resolution of species identification. Other tests that should be included are those that assessed for iron uptake, niacin accumulation, nitrate reduction, pyrazinamide sensitivity, and tellurite reduction. Also, the use of commercially prepared test media like the Tween 80 hydrolysis reagent should be considered to address the potential problem of batch variation among in-house prepared media, which could jeopardize the reproducibility of test results.

### 5.9.4 New *Mycobacterium*-specific Primers

Redesigning of the mycobacterial 16S primers or targeting alternative housekeeping genes or genes specific to the genus *Mycobacterium* (i.e. those involve in lipid metabolism) should be considered to address the specificity issue with nonmycobacterial actinobacteria and NTC.

### 5.9.5 Further Characterization of NTM Isolates

The NTM isolates should be further characterized using more robust methods, such as HPLC analysis of mycolic acids, to confirm their identities. In the case of HPLC lipid analysis, the chromatograms generated for the isolates can be compared against those deposited in available databases for homology-based identification. Determining the drug susceptibility profiles of the NTM isolates should also be carried out, in view of their role in human and animal infections.

# CHAPTER 6

# CONCLUSION

Using the method described by Parashar *et al.* (2004), several NTM species were successfully isolated from the soils of a cow farm in Kampar. The identities of these NTM species were preliminary confirmed based on the combination of their phenotypic and genotypic characteristics. The most possible species for the NTM isolates were *M. fortuitum*, *M. senegalense*, and *M. porcinum*, respectively. Interestingly, *Nocardia* sp. was isolated from an ex-tin-mining lake in one case. Upon evaluation of their health impacts, the identified NTM species are generally found to be pathogenic to humans under immune deficit or predisposing conditions, and can also cause infections in animal hosts. Since all three species were isolated from the cow farm, both the farmers and the animals are at risk of infection, subject to their immune and other physiological status. In the case of the former, wound and chronic lung infections of susceptible individuals are of great concern. The susceptibility of the latter to *M. senegalense* infection, if not monitored, could lead to considerable economic losses due to death of animals. In the event that more studies were to be performed on soils from this area, evaluation and use of a more suitable isolation method and improvement on current methods of NTM identification must be done. Further studies on similar locations outside Kampar should also be considered.

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# APPENDIX A

## APPARATUS AND CONSUMABLES

**Table A1: List of apparatus and their respective manufacturers**

| Apparatus | Manufacturer |
|---|---|
| Autoclave machine | Hirayama |
| Centrifuge machines | Sigma |
| Deionized water dispenser | Sartorius Stedim Biotech |
| Electrophoresis system | Hoefer |
| Filter units (0.2 µm) | Nalgene |
| Gel imaging system | Syngene Bio Imaging |
| Incubators | Memmert |
| Laminar air flow | Esco |
| Micropipettes (10 µl, 100 µl, 1000 µl) | Biohit |
| Microscope | Leica |
| Nanophotometer | Impen |
| PCR thermocycler | Biometra |
| Syringes | Cellotron |
| Syringe filters (0.2 µm) | Pall Life Science |
| Water bath | Memmert |

**Table A2: List of consumables and their respective manufacturers**

| Consumable | Manufacturer |
|---|---|
| Agar-agar powder | Merck |
| Agarose powder | Vivantis |
| Basic fuchsin | Merck |
| Calcium chloride ($CaCl_2$) | Gene Chem |
| Cetrimide | R & M Chemical |
| Crystal violet | Merck |
| Deoxyribonucleoside triphosphate (dNTP) mix | Fermentas |
| Dextrose | Rdeh |
| Dipotassium hydrogen phosphate ($K_2HPO_4$) | HmbG Chemicals |
| DNA ladder | Fermentas |
| Eggs | Tesco |
| Ethanol | Labmart |
| Ethidium bromide | Bio Basic |
| Ethylenediaminetetraacetic acid (EDTA) | Systerm |
| Gel purification kit | Stratec Molecular |
| Glacial acetic acid | Lab-scan |
| Glycerol | QRëC |
| Hydrochloric acid (HCl) | Merck |
| Hydrogen peroxide ($H_2O_2$) | HmbG Chemicals |
| Iodine | Merck |
| Luria-Bertani (LB) agar powder | Merck |
| LJ medium base powder | Merck |
| Magnesium sulfate ($MgSO_4$) | QRëC |
| Methylene blue | Systerm |
| Middlebrook 7H9 broth powder | Fisher Scientific |
| *Mycobacterium smegmatis* ATCC 607 | Microbiologics |
| Oleic acid-albumin-dextrose-catalase (OADC) | BD |
| Peptone | BD |
| Penicillin G | Bio Basic |
| Phenol | Merck |
| Phenol red | Nacalai Tesque |
| Potassium dihydrogen phosphate ($KH_2PO_4$) | QRëC |
| PCR primers | 1$^{st}$ Base |
| Safranin | Merck |
| SDS | Merck |
| NaCl | Merck |
| NaOH | Merck |
| *Taq* polymerase and PCR buffer | Fermentas |
| Tris base | Calbiochem |
| Tween 80 | Sigma-Aldrich |
| Urea | Systerm |